CONTRIBUTION DU RÉGULON SIGMA B À LA PATHOGENÈSE DE VARIANTS DE *STAPHYLOCOCCUS AUREUS* FORMANT DE PETITES COLONIES LORS D'INFECTIONS PULMONAIRES CHRONIQUES CHEZ LES PATIENTS ATTEINTS DE FIBROSE KYSTIQUE

par

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thèse présentée au Département de biologie en vue
de l'obtention du grade de docteur ès sciences (Ph.D.)

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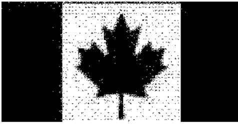

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Le 3 août 2012

le jury a accepté la thèse de Monsieur Gabriel Mitchell dans sa version finale.

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SOMMAIRE

Bien que la fibrose kystique soit fondamentalement causée par une défectuosité génétique, les infections microbiennes sont la plus grande cause de mortalité des gens qui en sont atteints. La bactérie *Staphylococcus aureus* est un des pathogènes les plus communs associés à cette maladie et est la cause d'infections persistantes et difficiles à traiter. Des souches variantes de *S. aureus* formant de petites colonies (les *small-colony variants* ou SCVs) sont fréquemment isolées des voies respiratoires des patients atteints de fibrose kystique lors d'infections bactériennes chroniques.

Mon projet de doctorat a consisté à déterminer les bases moléculaires de la persistance des infections pulmonaires à *S. aureus* chez les patients atteints de fibrose kystique et, plus particulièrement, le rôle des SCVs dans l'établissement d'infections chroniques. Mes principaux efforts de recherche ont été dirigés vers la compréhension du rôle de gènes et de phénotypes influencés par le facteur de transcription sigma alternatif sigma B dont l'activité est accrue chez les SCVs. Les mécanismes moléculaires par lesquels ce facteur de transcription influence la formation de biofilm, la persistance à l'intérieur des cellules de l'hôte et l'infection proprement dite ont été étudiés. Je me suis aussi intéressé à la compréhension des différents facteurs environnementaux et mécanismes moléculaires favorisant la présence et la persistance de *S. aureus* dans les poumons des patients fibrokystiques. Mes efforts de recherche ont également été dirigés vers l'élaboration d'antibiothérapies alternatives permettant de combattre les infections chroniques à *S. aureus*.

Mots-clés : *Staphylococcus aureus*, fibrose kystique, sigmaB, infections chroniques, pathogenèse, antibiothérapies.

À ma fille Lily, ma femme Émilie,
ma sœur Dorothée
& mes parents Line et Douglas

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LISTE DES ABRÉVIATIONS

ABC	*ATP-binding cassette*
ADN	acide désoxyribonucléique
Agr	*accessory gene regulator*
AIP	*auto-inducing peptide*
AMPc	adénosine monophosphate cyclique
ARN	acide ribonucléique
ATP	adénosine triphosphate
Bap	*biofilm-associated protein*
BHIg	*brain heart infusion* supplémenté de glucose
CFTR	*cystic fibrosis transmembrane conductance regulator*
ClfA	*clumping factor A*
CFU	*colony-forming unit* (unité formant des colonies)
CMI	concentration minimale inhibitrice
Eap	*extracellular adherence protein*
ENaC	*epithelial sodium (Na) channel*
FK	fibrose kystique
FnBPA	*fibronectin-binding protein A*
FnBPB	*fibronectin-binding protein B*
FnBPs	*fibronectin-binding proteins*
Hla	α-hemolysine
Hlb	β-hémolysine
Hld	δ-hémolysine
Ica	*intercellular adhesion*
IL-8	interleukine 8
MH	*mueller-hinton*

MSCRAMM	*microbial surface components recognizing adhesive matrix molecules*
NF-κB	*nuclear factor-kappa B*
NupC	*nucleoside permease C*
PCR	*polymerase chain reaction* (reaction de polymérisation en chaîne)
PIA	*polysaccharide intercellular adhesin*
PMSF	*phenylmethylsulfonyl fluoride*
Rot	*repressor of toxins*
RsbU	*regulator of sigma B U*
RsbV	*regulator of sigma B V*
RsbW	*regulator of sigma B W*
SarA	*staphylococcal accessory regulator A*
SARM	*S. aureus* résistant à la méthicilline
SarS	*staphylococcal accessory regulator S*
SarT	*staphylococcal accessory regulator T*
SCVs ou SCV	*small-colony variants* (variants à petites colonies)
SigB	*alternative sigma factor sigma B*
shRNA	*short hairpin RNA*
STX	triméthoprime-sulfaméthoxazole
TA	toxine-antitoxine
UDP	uridine diphosphate

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INTRODUCTION

1. La fibrose kystique

1.1. Manifestations cliniques

La fibrose kystique (FK) est une maladie génétique sévère, monogénique et récessive retrouvée en prédominance chez les populations caucasiennes de descendance européenne avec une incidence approximative de 1 sur 3000 naissances (Davis *et al.*, 1996). Elle est associée à une myriade de problèmes médicaux allant des infections sino-pulmonaires chroniques à des anormalités d'ordre gastro-intestinal. Cependant, les infections pulmonaires sont la plus problématique de ses caractéristiques cliniques puisque de 80 à 95% des patients FK succomberont à des déficiences respiratoires faisant suite à des infections bactériennes chroniques et à l'inflammation (Lyczak *et al.*, 2002).

1.2. Fondements moléculaires de la FK

1.2.1. Structure et fonctions du CFTR

La FK est causée par des mutations dans le gène *CFTR* (*cystic fibrosis transmembrane conductance regulator*). Bien qu'un très grand nombre de mutations causant la FK aient à ce jour été décrites, seulement quelques mutations sont retrouvées à des fréquences importantes

et la plus commune des mutations, une délétion de trois paires de bases encodant une phénylalanine en position 508 de la protéine, constitue 70% des allèles retrouvés chez les Caucasiens atteints de cette maladie (Gibson *et al.*, 2003).

Le CFTR est membre d'une famille de protéines transmembranaires qui sont des transporteurs de type ABC (*ATP-binding cassette*) (Hyde *et al.*, 1990). Cette protéine comprend deux domaines liant des nucléotides et deux domaines transmembranaires caractéristiques de cette famille de protéines ainsi qu'un domaine régulateur unique arborant plusieurs sites de phosphorylation. La phosphorylation dépendante de l'AMPc du domaine R module l'activité du canal (Ostedgaard *et al.*, 2001) et la liaison et l'hydrolyse de l'ATP par les domaines liants les nucléotides contrôle son ouverture (Gadsby et Nairn, 1999; Hanrahan et Wioland, 2004).

Le CFTR est exprimé à la surface apicale de plusieurs types de cellules épithéliales dont celles retrouvées dans les glandes sudoripares et salivaires, les poumons, le nez, le *vas deferens* des mâles, les canaux biliaires, le pancréas et les intestins. Cette protéine joue un rôle pivot dans la sécrétion de fluides par les tissus épithéliaux et, en plus d'être elle-même un canal chlorure, la liste des protéines avec lesquelles elle interagit en tant que régulateur de la conductance transmembranaire ne cesse de grandir et comprend des transporteurs, des protéines liées au cytosquelette apical des cellules épithéliales ainsi que d'autres canaux ioniques tels que le canal à sodium épithélial sensible à l'amiloride ou ENaC (*epithelial sodium (Na) channel*) (Hummler *et al.*, 1996).

1.2.2. Impact d'un CFTR défectueux sur la physiologie pulmonaire

Certaines études suggèrent un rôle important des glandes de la sous-muqueuse dans la pathophysiologie pulmonaire des patients atteints de la FK (Verkman *et al.*, 2003). Le CFTR est en effet hautement exprimé dans les glandes de la sous-muqueuse pulmonaire (Engelhardt *et al.*, 1993; Guggino, 1999) et la perte de la fonctionnalité du CFTR semble altérer la composition macromoléculaire et la viscosité des sécrétions glandulaires, et ainsi, affecter le dégagement mucociliaire pulmonaire (Jayaraman *et al.*, 2001; Knowles et Boucher, 2002; Verkman *et al.*, 2003). Une autre hypothèse, plus controversée, veut que la concentration en sel du liquide se retrouvant à la surface des poumons soit plus élevée chez les patients FK, ce qui aurait pour effet d'inactiver l'activité de certains peptides antimicrobiens (Gibson *et al.*, 2003; Smith *et al.*, 1996).

La plus évidente des défectuosités pulmonaires des patients FK semble explicable par un mauvais transport des sels à travers l'épithélium ainsi que par l'incapacité de l'organisme à hydrater correctement ses sécrétions. Chez ces patients, la sécrétion pulmonaire d'ions par le CFTR et les canaux ioniques dont l'activité dépend du CFTR est réduite ainsi que la quantité d'eau qui est normalement parallèlement relâchée. De plus, le canal ENaC responsable de la réabsorption pulmonaire du sodium et de l'eau (Hummler *et al.*, 1996) voit son activité augmentée en absence d'un CFTR fonctionnel (Jiang *et al.*, 2000). La combinaison de l'augmentation de la réabsorption et de la diminution de la sécrétion de l'eau a pour résultat un manque de fluides dans le système respiratoire des patients FK (Matsui *et al.*, 1998). Une des conséquences hypothétiques de la diminution du volume de liquide à la surface apicale de l'épithélium des poumons est l'interférence des fonctions ciliaires constituant normalement une barrière immunitaire innée importante en entraînant le mucus, ainsi que les microorganismes qui y sont prisonniers, vers l'extérieur. La mauvaise hydratation du mucus pourrait aussi contribuer au mauvais dégagement ciliaire pulmonaire par une augmentation de

sa viscosité. Durant les dernières étapes de la maladie, des plaques très denses de mucus sont retrouvées à la surface apicale de l'épithélium et les sécrétions pulmonaires deviennent stagnantes.

1.2.3. Association entre la fonctionnalité du CFTR et la susceptibilité aux infections bactériennes

Plusieurs modèles ont été proposés afin d'expliquer comment la perte de la fonction du CFTR augmente la susceptibilité des patients FK aux infections (Moskowitz *et al.*, 2005). La surface altérée des épithéliums pulmonaires FK pourrait promouvoir l'adhérence bactérienne (Zar *et al.*, 1995). Par exemple, l'asialo-GM1 est un ligand de *Pseudomonas aeruginosa*, de *Staphylococcus aureus* et d'*Haemophillus influenzae* dont l'expression est augmentée à la surface des cellules épithéliales pulmonaires FK (Saiman *et al.*, 1992; Saiman et Prince, 1993). Il est aussi possible que la réponse inflammatoire pulmonaire exagérée des patients FK promeut les infections bactériennes (Machen, 2006; Moskowitz *et al.*, 2005). Cependant, il est généralement accepté que le transport épithélial altéré des ions caractérisant la FK explique en grande partie la susceptibilité des patients aux infections, soit en inhibant l'activité des peptides antimicrobiens de l'hôte (Cole et Ganz, 2002; Smith *et al.*, 1996) ou en empêchant le dégagement mucociliaire des sécrétions pulmonaires (Matsui *et al.*, 1998). La rétention des plaques de mucus dans les voies respiratoires semble être un des premiers évènements prédisposant les gens ayant la FK aux infections pulmonaires chroniques par des pathogènes opportunistes, entre autres en favorisant leur croissance et leur métabolisme anaérobique (Worlitzsch *et al.*, 2002). Toutefois, selon d'autres études, la rétention des sécrétions pulmonaires pourrait être suffisante au déclenchement de l'inflammation même en absence d'infection, ce qui supporte l'hypothèse que l'inflammation pulmonaire pourrait tout de même précéder l'infection dans les stades précoces de la FK (Khan *et al.*, 1995; Tirouvanziam *et al.*, 2000).

1.2.4. Inflammation chronique chez les patients FK

L'inflammation locale commence tôt dans la vie des patients atteints de la FK et progresse jusqu'à contribuer à l'obstruction et à la destruction des voies respiratoires. Les sécrétions pulmonaires des gens atteints de la FK présentent souvent des concentrations élevées de neutrophiles, de cytokines et de chimioattractants, et cela, même chez les jeunes enfants et chez les adultes à l'état de santé stable ou présentant seulement des symptômes faibles de la maladie (Bonfield *et al.*, 1999). La sécrétion de cytokines et de chimioattractants par les cellules épithéliales entraîne probablement la migration initiale des neutrophiles dans les voies respiratoires. En plus de relâcher des médiateurs pro-inflammatoires, ces neutrophiles libèrent massivement des protéases. Ces protéases satureront les systèmes de défense antiprotéases, blesseront l'épithélium pulmonaire, altéreront la structure des voies respiratoires (Elizur *et al.*, 2008) et interféreront possiblement avec l'immunité innée de l'hôte (Tosi *et al.*, 1990). Parallèlement à la progression de la maladie, l'inflammation pulmonaire localisée sera de plus en plus associée avec une réponse inflammatoire systémique (Elizur *et al.*, 2008).

Les mécanismes liant la fonction anormale du CFTR, les infections bactériennes chroniques et l'inflammation pulmonaire ne sont pas complètement élucidés. Bien que certaines études suggèrent fortement que les infections bactériennes chroniques sont la cause majeure de l'inflammation rencontrée chez les patients FK (Armstrong *et al.*, 2005; Venkatakrishnan *et al.*, 2000), d'autres études supportent l'idée que l'épithélium pulmonaire FK a des caractéristiques pro-inflammatoires inhérentes et que l'inflammation pulmonaire pourrait se manifester d'une façon indépendante à l'infection (Hunter *et al.*, 2010; Joseph *et al.*, 2005; Rosenfeld *et al.*, 2001; Rubin, 2007; Teichgraber *et al.*, 2008; Tirouvanziam *et al.*, 2000; Tirouvanziam *et al.*, 2002; Vij *et al.*, 2009; Weber *et al.*, 2001). Il semble plus certain que l'inflammation pulmonaire FK est excessive et soutenue relativement aux stimuli infectieux. Par exemple, plusieurs modèles *in vitro* montrent que les cellules pulmonaires FK relâchent

une quantité accrue de médiateurs inflammatoires suite à une exposition à certains stimuli, soit par une dérégulation de l'activité du facteur de transcription NF-κB (Tirouvanziam *et al.*, 2002) et/ou tout simplement par un mauvais contrôle de la réponse immunitaire (Black *et al.*, 1998). Bien qu'il semble y avoir une variabilité considérable parmi les différents modèles cellulaires FK en ce qui a trait à leur réponse à des stimuli inflammatoires (Aldallal *et al.*, 2002), de plus hautes concentrations de neutrophiles et d'IL-8 ont été retrouvées dans les poumons des patients FK en comparaison à des sujets contrôles indépendamment de la présence ou de l'absence de pathogènes, ce qui supporte l'idée d'une réponse inflammatoire excessive et d'un contrôle défectueux des mécanismes de l'immunité (Nixon *et al.*, 2002).

En résumé, il semble que lors des premières infections rencontrées par les patients FK, les poumons recrutent très rapidement un très grand nombre de neutrophiles et sont la plupart du temps capables de contenir la menace bactérienne. Durant quelques années, les infections microbiennes sont souvent éradiquées et les colonisations bactériennes sont intermittentes. Cependant, les poumons FK ont tendance à réagir avec une réponse inflammatoire excessive aux stimulations bactériennes et cette réponse se maintient souvent bien après que l'infection ait été contrôlée. Ultimement, les facteurs immunitaires pulmonaires servant à contenir les infections bactériennes submergent les mécanismes de défense et les signaux inflammatoires initiateurs persistent. Tôt ou tard, la réponse inflammatoire devient excessive, dommageable pour le patient et pourrait même promouvoir les infections.

1.3. Microbiologie des voies respiratoires FK

1.3.1. Microflore et dynamique microbienne des patients FK

La colonisation des voies respiratoires des patients FK par les bactéries a habituellement lieu durant l'enfance et conduira à l'établissement d'infections chroniques qui provoqueront éventuellement des déficiences respiratoires et la mort (Harrison, 2007; Lyczak *et al.*, 2002). Les espèces bactériennes colonisant les voies respiratoires FK originalement décrites comprennent *P. aeruginosa*, *S. aureus*, *Haemophilus influenzae*, les bactéries du complexe des *Burkholderia cepacia* et *Stenotrophomonas maltophilia*, mais les plus récentes études démontrent que les poumons FK sont colonisés par des communautés polymicrobiennes beaucoup plus complexes et constituées de nombreuses autres espèces (Sibley et Surette, 2011). La prévalence des différentes espèces bactériennes varie en fonction de l'âge des patients d'une telle sorte que *H. influenzae* et *S. aureus* sont plus fréquents lors de la jeune enfance et que *P. aeruginosa* devient de plus en plus important comme les patients vieillissent (Harrison, 2007; Lyczak *et al.*, 2002).

P. aeruginosa et *S. aureus* sont encore et toujours des espèces bactériennes à hautes prévalences chez les patients FK (Canadian Cystic Fibrosis Foundation, 2009; Cystic Fibrosis Foundation, 2009; European Cystic Fibrosis Society, 2009). Parmi les autres microorganismes retrouvés dans les voies respiratoires FK sont les mycobactéries, des virus pathogènes, des champignons (*p. ex. Aspergillus fulmigatus*) et des levures (*p. ex. Candida albicans*) (Harrison, 2007; Moskowitz *et al.*, 2005). La contribution précise de différentes espèces microbiennes à la progression de la maladie demeure indéterminée, et cela même pour les microorganismes les moins fréquemment isolés.

1.3.2. Phénotypes microbiens impliqués dans le développement d'infections persistantes et récalcitrantes aux antibiothérapies

Le problème toujours grandissant des bactéries résistantes aux antibiotiques en médecine humaine et vétérinaire n'épargne pas les patients FK. En effet, des bactéries résistantes aux antibiotiques sont fréquemment isolées d'échantillons cliniques FK (George *et al.*, 2009; Parkins et Elborn, 2010) et la résistance des pathogènes FK aux thérapies antibiotiques est associée à la morbidité de cette maladie (Chmiel et Davis, 2003; Lyczak *et al.*, 2002). En plus d'être explicable par l'utilisation d'un arsenal d'antibiotiques de plus en plus désuet (Shah, 2005; Talbot *et al.*, 2006; Wenzel *et al.*, 2005), cette résistance aux traitements antimicrobiens semble être favorisées par l'infection à long terme des voies respiratoires, permettant non seulement aux pathogènes de contourner les réactions immunitaires de l'hôte, mais aussi de s'adapter aux antibiothérapies (Goerke et Wolz, 2010; Hogardt et Heesemann, 2010). Ainsi, plusieurs mécanismes diminuant la susceptibilité des bactéries aux antimicrobiens sont activés lors d'infections FK telles l'expression de pompes à efflux et la mutation des molécules ciblées par les antibiotiques (Hoiby *et al.*, 2010), la formation de cellules dormantes/persistantes (Mulcahy *et al.*, 2010) et de variants à petites colonies (Goerke et Wolz, 2010; Proctor *et al.*, 2006; Schneider *et al.*, 2008) ainsi que la croissance en biofilm (Hoiby *et al.*, 2010; Wagner et Iglewski, 2008), reconnu pour son rôle lors d'infections persistantes (Galli *et al.*, 2007; Stewart, 2002). Les bactéries poussant en biofilm favoriseraient la persistance dans les voies respiratoires FK parce qu'elles semblent protégées physiquement du système immunitaire et sont jusqu'à 1000 fois plus résistantes aux antimicrobiens comparativement à leur contrepartie planctonique (Costerton *et al.*, 1999; Davies et Bilton, 2009; George *et al.*, 2009).

Par exemple, des changements adaptatifs substantiels sont observés chez *P. aeruginosa* lors d'infections chroniques des voies respiratoires FK (Foweraker *et al.*, 2005; Smith *et al.*, 2006;

Sriramulu *et al.*, 2005). Ces changements entrainent les bactéries à se diversifier et à montrer des caractéristiques phénotypiques différentes de celles retrouvées chez les isolats environnementaux. L'adaptation de *P. aeruginosa* durant les infections persistantes des voies respiratoires FK mène souvent à la résistance aux antibiotiques, à la surproduction d'alginate et à des changements profonds du métabolisme. En général, *P. aeruginosa* adoptera avec le temps un comportement moins agressif en réprimant l'expression de plusieurs de facteurs de virulence et d'autres produits immunostimulateurs, poussera sous forme de biofilms et adaptera son métabolisme à l'environnement microaérobique formé par les plaques de mucus (Hogardt et Heesemann, 2010). Aussi, les souches non mucoïdes colonisant initialement les patients FK pourront acquérir le phénotype mucoïde qui montre une résistance à plusieurs antibiotiques et qui est difficile à éradiquer des voies respiratoires (George *et al.*, 2009). D'autres variantes phénotypiques de *P. aeruginosa* ont été isolées des poumons FK telles les cellules persistantes (Mulcahy *et al.*, 2010) et les souches variantes à petites colonies (Haussler *et al.*, 1999; Schneider *et al.*, 2008). Il est suggéré que l'hypermutabilité des souches FK de *P. aeruginosa* accélère le développement de résistances aux antibiotiques et l'acquisition des adaptations requises à la persistance de cette bactérie chez l'hôte (Macia *et al.*, 2005).

1.3.3. Interactions microbiennes entre *P. aeruginosa* et *S. aureus*

L'idée que les microorganismes ont la capacité de sentir des signaux moléculaires au-delà de la frontière des espèces et que ce phénomène influence le développement des communautés microbiennes ainsi que la virulence des pathogènes durant l'infection est acceptée (Ryan et Dow, 2008). Ces mécanismes de communication interspécifique semblent encore plus pertinents à l'étude des biofilms puisque les biofilms peuvent être considérés comme des communautés complexes intégrants fréquemment plusieurs espèces de microbes (Stoodley *et al.*, 2002). Les biofilms sont d'une importance majeure dans le contexte de la FK (Davies et

Bilton, 2009) et il est de plus en plus évident que les très nombreuses espèces bactériennes colonisant les voies respiratoires FK interagissent (Bakkal *et al.*, 2010; Biswas *et al.*, 2009; Chattoraj *et al.*, 2010; Duan *et al.*, 2003; Hoffman *et al.*, 2006; Mashburn *et al.*, 2005; McAlester *et al.*, 2008; Qazi *et al.*, 2006; Riedel *et al.*, 2001; Ryan *et al.*, 2008; Sibley *et al.*, 2008; Sibley et Surette, 2011; Weaver et Kolter, 2004; Yang *et al.*, 2011).

Une attention particulière a été portée à l'interaction entre *P. aeruginiosa* et *S. aureus* étant donné leur haute prévalence et le fait que ces bactéries sont souvent co-isolées des voies respiratoires FK (Harrison, 2007; Hoffman *et al.*, 2006). Par le passé, des interactions synergiques ont été proposées entre ces deux espèces et il a été suggéré que *S. aureus* sensibilise les poumons aux infections à *P. aeruginosa* (Lyczak *et al.*, 2002). Cependant, certaines études récentes suggèrent plutôt que des interactions antagonistes existent entre ces deux pathogènes (Mashburn *et al.*, 2005; Palmer *et al.*, 2005). Il est maintenant connu que *P. aeruginosa* interagit avec *S. aureus* en utilisant plusieurs exoproduits tels que le 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) (Hoffman *et al.*, 2006) ainsi que d'autres inhibiteurs de la chaîne de transport des électrons (Biswas *et al.*, 2009; Voggu *et al.*, 2006), l'endopeptidase staphylolytique LasA (Kessler *et al.*, 1993) et certaines de ses homosérines lactones (Kaufmann *et al.*, 2005; Qazi *et al.*, 2006). Aussi, bien que peu d'études aient adressé l'influence de *S. aureus* sur *P. aeruginosa*, il est probable que *P. aeruginosa* soit capable de sentir et de réagir à la présence de *S. aureus* dans son environnement en augmentant la production de facteurs de virulence (Korgaonkar et Whiteley, 2011).

1.4. Nouvelles approches thérapeutiques considérées dans le traitement des infections microbiennes FK

L'humanité a actuellement une capacité limitée à combattre les infections microbiennes à cause des pathogènes résistants aux antibiotiques. Le nombre d'infections nosocomiales et acquises dans la communauté impliquant des souches résistantes à plus d'une classe d'antibiotiques devient un problème de santé publique inquiétant. La raison principale de l'émergence de ce problème de multirésistance aux antibiotiques vient du manque d'innovation actuel dans l'élaboration d'antibiothérapies et de l'utilisation d'un arsenal d'antibiotiques rendu désuet par la dispersion des résistances bactériennes de par le monde (Shah, 2005; Talbot *et al.*, 2006). L'identification de nouvelles cibles antibiotiques et le développement de nouvelles approches thérapeutiques pourraient grandement jouir d'une plus grande compréhension de la pathogenèse bactérienne autant au niveau d'espèces singulières qu'au niveau des communautés polymicrobiennes. Plusieurs nouvelles approches thérapeutiques prenant en compte la pathogenèse et l'adaptation des pathogènes FK sont envisagées. Plus spécifiquement, plusieurs approches ciblant les biofilms bactériens sont considérées telle l'élaboration de traitements contre certains phénotypes persistants (Batoni *et al.*, 2011; Hu *et al.*, 2010; Lewis, 2007; Nguyen *et al.*, 2009a; Ooi *et al.*, 2010) ou altérant la virulence (Bjarnsholt *et al.*, 2005; Hentzer *et al.*, 2003; Njoroge et Sperandio, 2009; Rasko et Sperandio, 2010; Rasmussen *et al.*, 2005; Song *et al.*, 2010; Wu *et al.*, 2011) ainsi que l'utilisation de combinaisons de composés synergiques afin d'augmenter l'efficacité de thérapies antimicrobiennes existantes (Baltch *et al.*, 2008; Dales *et al.*, 2009; George *et al.*, 2009; Hoiby, 2011; Nguyen *et al.*, 2009b). Comme souligné dans la littérature (Mohtar *et al.*, 2009), de nombreuses nouvelles molécules possédant des activités antimicrobiennes attendent potentiellement d'être découvertes dans le monde végétal. De plus, plusieurs produits de plantes atténuent la virulence de certains pathogènes humains et pourraient donc être utilisés afin de moduler la virulence et l'habileté de la bactérie à s'adapter à l'hôte. D'autres produits de plantes augmentent l'activité de certains antibiotiques en inhibant des pompes à efflux

bactériennes ou en déstabilisant les membranes (voir Annexe 2, Résumé 5). Il est donc possible que certaines molécules en provenance du monde végétal puissent servir d'échafaudage structural au développement de nouveaux composés utilisables dans le traitement des infections pulmonaires FK.

Les saponines sont produites de façon constitutive et jouent un rôle important dans la réponse des plantes contre les insectes et microbes pathogènes. Ces molécules glycosylées peuvent être divisées en trois grands groupes selon la structure de leur portion aglycone: les triterpenoïdes, les stéroïdes et les glycoalkaloïdes stéroïdales (Osbourn, 1996). La principale saponine retrouvée chez la tomate est l'α-tomatine et présente une activité antimicrobienne contre les levures et une variété de microbes (Bednarek et Osbourn, 2009; Friedman, 2002; Osbourn, 1996; Sandrock et Vanetten, 1998). Cependant, plusieurs champignons résistent à l'activité de cette molécule en produisant les tomatinases, des enzymes capables d'inactiver la tomatine (Martin-Hernandez *et al.*, 2000; Roddick, 1974; Ruiz-Rubio *et al.*, 2001). Il a en effet été démontré que ces enzymes constituent un déterminant de la virulence de certains champignons phytopathogènes lors de l'infection de plantes produisant la tomatine (Bouarab *et al.*, 2002; Maor et Shirasu, 2005; Martin-Hernandez *et al.*, 2000). Par exemple, *Fusarium oxysporum* f. sp. *lycopersici* scinde l'α-tomatine en portions aglycone (tomatidine) et lycotétraose, lesquelles montrent une activité antifungique nulle ou considérablement diminuée contre ce pathogène (Ruiz-Rubio *et al.*, 2001; Simons *et al.*, 2006). Le spectre d'activité antimicrobienne des saponines et de leurs dérivés est peu caractérisé et il demeure possible que ces molécules puissent un jour être utilisées pour combattre les infections chez l'humain.

2. Pathogenèse de *S. aureus* infectant les patients FK

2.1. Importance des infections pulmonaires à *S. aureus* chez les patients FK

S. aureus est un pathogène humain Gram-positif opportuniste associé à un taux de mortalité considérable dans les hôpitaux (Talbot *et al.*, 2006). Cette bactérie cause un impressionnant spectre de maladies, est capable d'infecter plusieurs hôtes et organes et a la capacité d'engendrer à la fois des infections fatales et chroniques (Archer, 1998; Goerke et Wolz, 2010). Approximativement 30% des humains transportent *S. aureus* d'une façon persistante et asymptomatique dans leur nasopharynx ou d'autres sites corporels. L'hypothèse est qu'un processus de coévolution ait mené à un état de tolérance mutuelle, mais que, lors du débalancement de l'interaction hôte-pathogène, des infections invasives peuvent avoir lieu. Alors qu'il est possible que certaines souches soient prédisposées à coloniser et/ou infecter l'humain, il semble que l'ensemble des souches de *S. aureus* possède la capacité de devenir invasive sous certaines circonstances (*p. ex.* la présence de traits de susceptibilité chez l'hôte) (van Belkum *et al.*, 2009). Le traitement des infections à *S. aureus* est sérieusement compromis par la résistance de cette bactérie aux antibiotiques qui s'est répandue parmi les espèces de staphylocoques (Witte *et al.*, 2008). Les *S. aureus* résistants à la méthicilline (SARM), bien connus pour causer des problèmes dans les hôpitaux, présentent maintenant un phénotype de multirésistance aux antibiotiques et causent même des problèmes dans la communauté (Chambers et Deleo, 2009).

La contribution de *S. aureus* à la progression de la maladie chez les patients FK est moins évidente que celle de *P. aeruginosa*. Même si la présence de cette bactérie dans les voies respiratoires inférieures est considérée comme étant représentative d'une situation pathologique, des questions demeurent concernant l'impact réel de *S. aureus* sur l'état du

patient (Goss et Muhlebach, 2011; Kahl, 2010; Lyczak *et al.*, 2002). Bien que certaines études supportent l'hypothèse que *S. aureus* contribue à la détérioration de l'état de santé et à l'inflammation pulmonaire chez le patient FK (Albus *et al.*, 1988; Armstrong *et al.*, 1997; Armstrong *et al.*, 2005; Hudson *et al.*, 1993; Ranganathan *et al.*, 2011; Sagel *et al.*, 2009), il est toujours possible que le traitement des infections à *S. aureus* puisse conduire à une acquisition précoce de *P. aeruginosa* ou même que les infections à *S. aureus* aient un effet bénéfique sur la fonction pulmonaire des patients (Lyczak *et al.*, 2002; Mayer-Hamblett *et al.*, 2007). Cependant, les plus récentes études indiquent que la prévalence de *P. aeruginosa* est actuellement en diminution tandis que celle de *S. aureus* augmente aux États-Unis, et cela autant pour les souches susceptibles que pour les souches résistantes à la méthicilline (Razvi *et al.*, 2009). De plus, la persistance de souches SARM dans les voies respiratoires des patients FK a été associée avec une diminution de la survie et avec une détérioration plus rapide des voies respiratoires (Dasenbrook *et al.*, 2010; Dasenbrook *et al.*, 2008). La raison(s) pour laquelle les SARM ont un plus grand impact sur l'état de santé du patient reste encore inconnue, mais il est possible que la multirésistance aux antibiotiques et la virulence altérée de ces souches soient des facteurs impliqués (Molina *et al.*, 2008; Pozzi *et al.*, 2012). Indépendamment de sa prévalence relative à celle d'autres pathogènes, il a été démontré que *S. aureus* persiste souvent dans les voies respiratoires des patients FK pour plusieurs mois ou années et est la cause d'infections récalcitrantes aux traitements antibiotiques (Goerke et Wolz, 2010; Kahl, 2010).

2.2. Contrôle de l'expression des facteurs de virulence chez le *S. aureus* prototypique

2.2.1. Rôle et fonction des facteurs de virulence dans la pathogenèse des infections à *S. aureus*

Les facteurs de virulence font partie de systèmes bactériens adaptatifs ou accessoires impliqués dans la pathogenèse et dans la survie de la bactérie à l'environnement hostile que représente l'hôte. Les facteurs de virulence peuvent soit être présentés à la surface de la bactérie ou relâchés dans le milieu environnant et permettent aux pathogènes d'éviter le système immunitaire, d'adhérer aux cellules et à la matrice extracellulaire de l'hôte, de se répandre dans l'organisme ainsi que de dégrader cellules et tissus afin de se nourrir et de se protéger. La présence de plusieurs gènes encodant des facteurs de virulence dans le génome de *S. aureus* explique potentiellement l'habileté qu'a cette bactérie à causer un large spectre de maladies (Archer, 1998; Novick, 2003).

2.2.2. Programme temporel et stratégies de régulation de l'expression des facteurs de virulence

L'expression des gènes impliqués dans la pathogenèse est étroitement contrôlée par des réseaux complexes de régulation qui permettent aux bactéries de produire les facteurs de virulence en fonction de la densité de leur population (*c.-à-d.* le *quorum-sensing*) et de leur environnement. Les réseaux de régulation de la virulence de *S. aureus* comprennent plusieurs systèmes de transduction à deux composants tels que le système *agr* de *quorum-sensing* ainsi que plusieurs facteurs de transcription tels que SarA et le facteur sigma alternatif sigma B (Bronner *et al.*, 2004; Novick, 2003). Vraisemblablement, l'activité d'un ensemble spécifique

de régulateurs de la virulence permet l'expression des facteurs requis à des étapes précises de l'infection ou pour différents types d'infections.

In vitro, la production des exoprotéines suit un programme temporel spécifique chez les souches prototypes où la production d'adhésines et de protéines attachées à la surface bactérienne précède celle des hémolysines, des protéases et d'autres enzymes de dégradation (Dunman *et al.*, 2001; Novick, 2003). Tel qu'illustré à la Figure 1, cette transition dans le patron d'expression des facteurs de virulence corrèle avec l'augmentation de la densité de la population bactérienne et l'activation du système *agr*, connu pour réprimer l'expression de plusieurs protéines liées à la surface bactérienne et pour augmenter celle de certaines protéines sécrétées (Dunman *et al.*, 2001; Novick *et al.*, 1993). Cependant, plusieurs signaux environnementaux autres que celui découlant de la densité bactérienne affectent la production des facteurs de virulence : le niveau énergétique ou de certains métabolites biosynthétiques (McNamara et Proctor, 2000; Somerville *et al.*, 2002), ainsi que le potentiel hydrogène, la force ionique et la présence de concentrations sous-inhibitrices d'éthanol et de certains antibiotiques (Herbert *et al.*, 2001; Novick, 2003) n'en sont que quelques exemples. De plus, la présence de différents gènes encodant des superantigènes dans le génome de certaines souches est aussi un facteur influençant l'expression des facteurs de virulence chez *S. aureus* (Vojtov *et al.*, 2002).

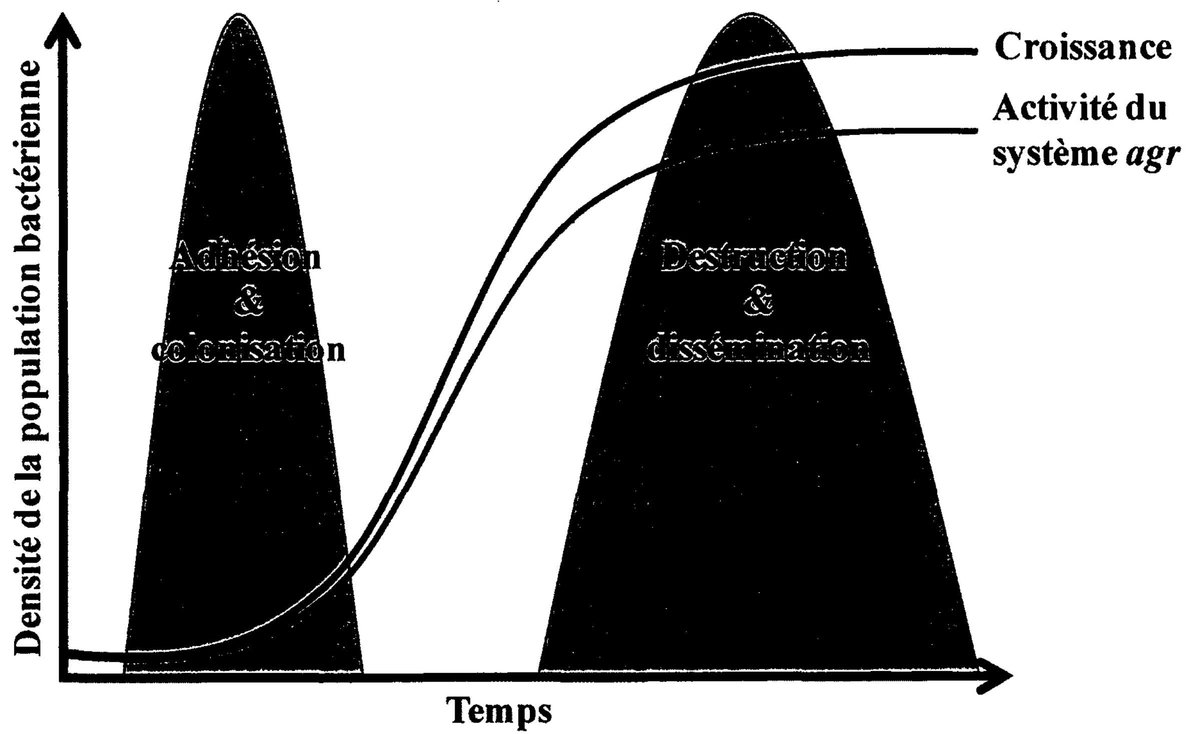

FIGURE 1. Programme temporel de l'expression des facteurs de virulence. Parallèlement à la croissance bactérienne d'une souche prototype de *S. aureus*, le système *agr* s'active et des facteurs de virulence intervenant potentiellement dans la destruction des tissus de l'hôte et la dissémination des infections, plutôt que l'adhésion et la colonisation, sont exprimés. Inspiré de Novick (2003).

2.2.3. Le système *agr*

Le système *agr* est un locus de régulation globale contrôlant réciproquement l'expression de certaines protéines associées à la surface et de protéines sécrétées par un circuit typique d'autoactivation (Morfeldt *et al.*, 1988; Novick, 2003; Peng *et al.*, 1988). Bien que des systèmes d'autoactivation similaires à *agr* et reposants sur l'utilisation de peptides extracellulaires inducteurs existent chez la plupart des bactéries Gram-positif (Kleerebezem *et*

al., 1997), le système *agr* des staphylococcies est unique, car il utilise un ARN régulateur en tant qu'effecteur au lieu de strictement utiliser un facteur protéique (Novick et Geisinger, 2008).

Le locus *agr* est composé de 5 gènes et produit deux transcrits divergents, RNAII et RNAIII, lesquels sont sous le contrôle de deux promoteurs distincts (P2 et P3). RNAII encode AgrB, AgrD, AgrC et AgrA. AgrC et AgrA sont respectivement une protéine de détection et de régulation de la réponse composant un système de transduction à deux composants. Le motif N-terminal d'AgrD cible le propeptide vers la membrane où sa région C-terminale interagira spécifiquement avec AgrB. AgrB catalysera vraisemblablement la coupure de l'extrémité C-terminale et la formation d'un groupement thiolactone. La partie N-terminale du propeptide sera coupée par la signal-peptidase SpsB. Le peptide auto-inducteur (ou *auto-inducing peptide*, AIP) résultant consiste en 7 à 9 résidus et contient le groupement thiolactone nécessaire à sa fonctionnalité. La liaison du peptide auto-inducteur à AgrC entraine l'autophosphorylation d'AgrC et, ultimement, la phosphorylation d'AgrA. AgrA sous sa forme phosphorylée a la capacité de lier et de transactiver les promoteurs P2 et P3. AgrA pourrait aussi interagir avec SarA à la région intergénique du locus *agr* puisqu'il a été démontré que SarA augmente l'activité transcriptionnelle induite par AgrA aux deux promoteurs du locus. RNAIII est un ARN de 514 nucléotides présentant une structure secondaire complexe et qui encode le peptide appelé δ-hémolysine. La δ-hémolysine n'a aucune fonction régulatrice connue, mais semble influencer la formation du biofilm. L'expression de RNAIII a des effets multiples autant au niveau transcriptionnel que traductionnel. Il a par exemple été montré que RNAIII contrôle le recrutement de la machinerie traductionnelle sur des ARNm spécifiques par un mécanisme de complémentarité antisens. Cependant, pendant que RNAIII semble réguler directement la traduction de transcrits spécifiques, il est probable que son effet sur la transcription soit indirect et implique des facteurs protéiques intermédiaires (Bronner *et al.*, 2004; Novick et Geisinger, 2008). Bien qu'il a été initialement montré que l'expression de RNAIII rétablit l'ensemble des fonctions

agr dans un mutant *agr* (Novick *et al.*, 1993), il semble que l'expression de certains gènes (surtout associés à des fonctions métaboliques) soit influencée par le système *agr* d'une façon indépendante de RNAIII (Queck et al., Mol cell, 2008). Toutefois, le système *agr* semble bel et bien réguler les facteurs de virulence par l'activité de cet ARN régulateur (Novick et Geisinger, 2008; Queck *et al.*, 2008).

Le système *agr* est activé lorsque la concentration extracellulaire de l'AIP atteint un certain seuil critique, ce qui est directement lié à une densité cellulaire déterminée. Il y aura alors une augmentation exponentielle de la concentration d'AIP et, rapidement, une réponse sera produite au niveau de l'ensemble de la population (*c.-à-d.* qu'il y aura atteinte d'un *quorum-sensing*). Cette réponse mènera à l'expression de plusieurs exoprotéines et à la répression des protéines de surface (Bronner *et al.*, 2004; Novick et Geisinger, 2008). Le mécanisme d'autoactivation du système *agr* est présenté à la Figure 2.

L'activité du système *agr* semble pouvoir être un facteur important à la pathogenèse de la bactérie *in vivo* puisque des mutants *agr* sont atténués dans une variété de modèles d'infections expérimentales animales (Abdelnour *et al.*, 1993; Cheung *et al.*, 1994; Gillaspy *et al.*, 1995; Mayville *et al.*, 1999; Wright *et al.*, 2005), mais il est bien de garder à l'esprit que le système *agr* semble sous-activé lors de certaines infections telles que les infections pulmonaires chez les patients FK (Goerke et Wolz, 2004), la colonisation chronique de cathéters (Rothfork *et al.*, 2003; Yarwood et Schlievert, 2003) et l'infection prolongée de tissus mous (Schwan *et al.*, 2003).

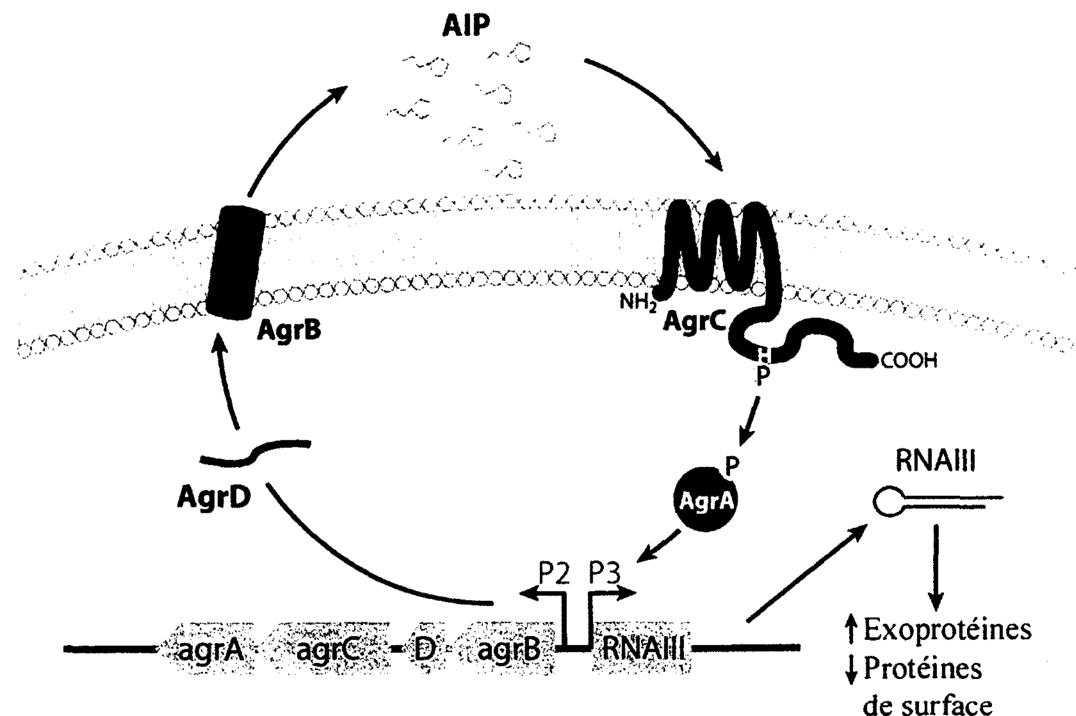

FIGURE 2. Organisation génétique et mécanisme d'autoactivation du système *agr*. Le système *agr* est un système de transduction à deux composants activé par un mécanisme de *quorum-sensing*. L'accumulation d'un peptide auto-inducteur dans le milieu extracellulaire mène à l'activation du système *agr* et à la modulation du programme d'expression génétique et protéique de la bactérie. L'activation du système *agr* conduit à la production de plusieurs exoprotéines et à la répression de certaines protéines de surface. Modifié de Novick et Geisinger (2008).

2.2.4. Le facteur de transcription sigma alternatif sigma B

Chez les eubactéries, la reconnaissance des promoteurs par le complexe protéique de la polymérase à ARN implique un facteur sigma. En addition au facteur sigma constitutif (ou *housekeeping*), des facteurs sigma alternatifs avec des spécificités de promoteurs précises

seront habituellement présents chez la bactérie. La disponibilité de ces facteurs sigma pour lier la polymérase à ARN altèrera globalement le programme transcriptionnel de la bactérie en réponse à des changements de conditions environnementales (Gruber et Gross, 2003). Par exemple, la réponse à certains stress est contrôlée par le facteur de transcription sigma alternatif sigma B chez *B. subtilis* et certaines autres bactéries Gram-positif par un mécanisme de transduction de signaux impliquant des interactions protéine-protéine régulées par phosphorylation (Price, 2002). Chez *S. aureus* et d'autres agents pathogènes Gram-positif, SigB régule l'expression des facteurs de virulence en plus d'être impliqué dans la réponse à différents stress (Bischoff *et al.*, 2004; Chan *et al.*, 1998; Gertz *et al.*, 1999; Giachino *et al.*, 2001; Horsburgh *et al.*, 2002). L'activité SigB de *S. aureus* est clairement détectable durant la phase de croissance exponentielle (Giachino *et al.*, 2001) et soutient l'expression de gènes tels que *fnbA* en phase de croissance exponentielle précoce tout en réprimant l'expression d'autres gènes encodant des exoprotéines en phase de croissance exponentielle tardive (Bischoff *et al.*, 2004). SigB peut aussi être activé par différents stress environnementaux tels le stress ionique, le choc alcalin et le choc thermique (Pane-Farre *et al.*, 2006; Pane-Farre *et al.*, 2009) et bien que controversé, possiblement la déplétion énergétique (Pane-Farre *et al.*, 2006; Proctor et von Humboldt, 1998; Senn *et al.*, 2005b).

Toujours chez *S. aureus*, l'activité de SigB est régulée par un mécanisme post-traductionnel impliquant RsbU, RsbV et RsbW (Pane-Farre *et al.*, 2009; Senn *et al.*, 2005b). Le facteur antisigma RsbW peut former des complexes protéiques mutuellement exclusifs avec SigB et RsbV. RsbV est normalement sous une forme phosphorylée inactive due à l'activité kinase de RsbW, ce qui l'empêche de se complexer avec RsbW, laissant ce dernier libre d'interagir et de séquestrer SigB. Lorsque séquestré par RsbW, SigB est incapable de se lier à la polymérase à ARN afin de former l'holoenzyme. Lors de stress, RsbU est activé et déphosphoryle RsbV, qui libérera alors SigB de l'emprise de RsbW. Le mécanisme de régulation post-traductionnel de SigB ainsi que l'impact de son activation sur le programme transcriptionnel de la bactérie sont illustrés à la Figure 3. Contrairement à *B. subtilis*, le mécanisme exact d'activation de

RsbU est méconnu chez *S. aureus* (Pane-Farre *et al.*, 2009). D'autres facteurs pourraient influencer l'activation de SigB (Senn *et al.*, 2005b).

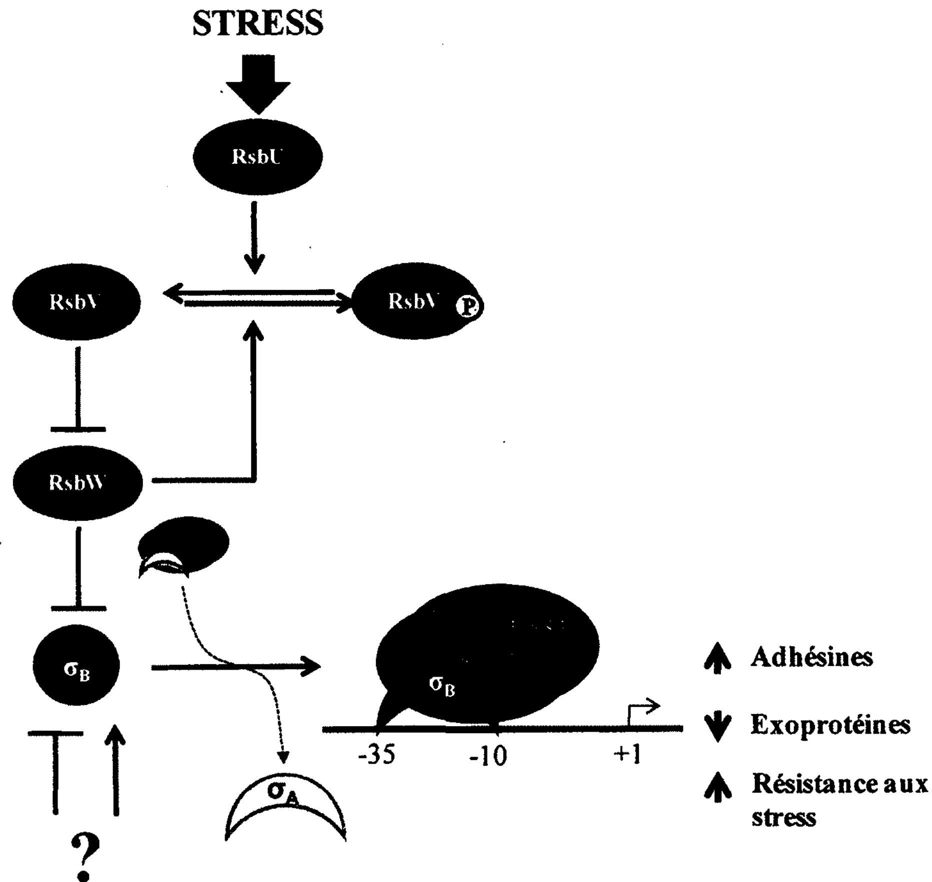

FIGURE 3. Mécanismes de régulation post-traductionnels de SigB. L'activation de SigB et sa liaison à la polymérase à ARN entraînera, soit directement ou indirectement, une expression de plusieurs protéines de surface et de gènes impliqués dans la réponse aux stress ainsi que la répression de certaines exoprotéines. Le mode d'activation de RsbU est encore inconnu et il est probable que d'autres facteurs influencent l'activation de SigB, comme souligné par le point d'interrogation.

Il a été démontré que SigB contrôle positivement et négativement l'expression de 198 et 53 cadres de lecture ouverts chez *S. aureus*, respectivement. Des gènes impliqués dans divers processus cellulaires tels que le métabolisme de la paroi bactérienne, des voies métaboliques intermédiaires, des voies de signalisation ainsi que des facteurs de virulence sont représentés dans le régulon SigB (Bischoff *et al.*, 2004). La plupart des gènes dont l'expression est augmentée par SigB sont précédés par une séquence nucléotidique s'apparentant à la séquence consensus des promoteurs SigB chez *B. subtilis* (Bischoff *et al.*, 2004). SigB est aussi connue pour influencer l'expression de plusieurs autres régulateurs globaux de la virulence tel que SarA (Bischoff *et al.*, 2001; Deora *et al.*, 1997; Manna *et al.*, 1998), SarS (Tegmark *et al.*, 2000), Rot (Hsieh *et al.*, 2008) et RNAIII (Bischoff *et al.*, 2001; Horsburgh *et al.*, 2002), ce qui suggère que SigB influence indirectement l'expression de certains gènes. Différentes études montrent que SigB est impliqué dans la résistance à différents stress tels que les chocs thermiques, les stress oxydatifs, les champs électriques, la pression hydrostatique, les pH acides et alcalins, les UV et la présence de certains antibiotiques (Cebrian *et al.*, 2009; Chan *et al.*, 1998; Gertz *et al.*, 1999; Giachino *et al.*, 2001; Horsburgh *et al.*, 2002; Inose *et al.*, 2006; Rice *et al.*, 2004; Schulthess *et al.*, 2009; Singh *et al.*, 2003; Tamber *et al.*, 2010). De plus, SigB module l'expression de différents facteurs de virulence tels que des adhésines (Cheung *et al.*, 2009; Entenza *et al.*, 2005), la capsule (Meier *et al.*, 2007; Schulthess *et al.*, 2009), des hémolysines (Herbert *et al.*, 2010; Karlsson-Kanth *et al.*, 2006), des gènes de pigmentation (Herbert *et al.*, 2010; Karlsson-Kanth *et al.*, 2006), des superantigènes (Kusch *et al.*, 2011) ainsi que des protéases, lipases et nucléases extracellulaires (Herbert *et al.*, 2010; Karlsson-Kanth *et al.*, 2006; Kiedrowski *et al.*, 2011; Lauderdale *et al.*, 2009; Schulthess *et al.*, 2011).

Bien que SigB influence la formation de biofilm (Herbert *et al.*, 2010; Houston *et al.*, 2011; Marti *et al.*, 2010) et l'interaction avec les cellules hôtes (Haslinger-Loffler *et al.*, 2005; Moisan *et al.*, 2006; Nair *et al.*, 2003), le rôle de SigB dans la pathogenèse de *S. aureus* demeure incertain puisque la virulence de mutants *sigB* n'est pas altérée dans plusieurs modèles d'infection en comparaison à leur contrepartie sauvage (Depke *et al.*, 2012).

Toutefois, l'activité de SigB semble importante dans l'établissement d'infections associées à des cathéters et dans la formation d'infections d'organes multiples (Lorenz *et al.*, 2008) ainsi que dans la persistance de l'infection dans un modèle d'arthrite septique induite (Jonsson *et al.*, 2004). SigB a aussi été associé à une augmentation de la mortalité dans un modèle murin de sepsie (Herbert *et al.*, 2010).

En amont de l'opéron *rsbUVW-sigB* se trouve les gènes *mazE* et *mazF* qui encodent un système toxine-antitoxine (TA) (Fu *et al.*, 2007; Mittenhuber, 1999). Les systèmes TA consistent habituellement en une antitoxine labile qui lie et inactive une toxine plus stable, et pourraient être impliqués dans la mort bactérienne programmée, l'interruption transitoire du métabolisme bactérien, la stabilisation de certaines sections du chromosome, la résistance aux phages et l'émergence de cellules persistantes (Aizenman *et al.*, 1996; Gerdes *et al.*, 2005; Magnuson, 2007). Il a aussi été démontré que *mazE* et *mazF* peuvent être cotranscrits avec le restant de l'opéron *sigB* et que ce transcrit est inductible lors de différents stress tels les chocs thermiques, ioniques et l'exposition à des antibiotiques (Fu *et al.*, 2007; Senn *et al.*, 2005b). Le promoteur en amont de *mazEF* (P_{mazE}) est essentiel à l'atteinte d'une activité SigB maximale (Donegan et Cheung, 2009). À la différence des systèmes TA typiques, MazEF n'est pas contrôlé par autorépression, mais l'activité de P_{mazE} est réprimée par SigB et activée par SarA (Donegan et Cheung, 2009). ClpPC est l'unité fonctionnelle dégradant l'antitoxine MazE, assurant que MazF est activé lorsque la production de MazE est interrompue (Donegan *et al.*, 2010). MazF est une endoribonucléase séquence-spécifique coupant des ARNm, inhibant la synthèse protéique et menant à un arrêt de croissance rapide (Fu *et al.*, 2007; Fu *et al.*, 2009). Il a aussi été démontré que MazF inactive sélectivement certains ARNm, évitant certains autres transcrits, sous certaines conditions (Fu *et al.*, 2009). Une étude suggère que MazEF pourrait même jouer un rôle dans la régulation de l'expression des facteurs de virulence et, conséquemment, dans la pathogenèse de *S. aureus* (Zhu *et al.*, 2009).

2.2.5. Autres systèmes de transduction à deux composants et facteurs de transcription contrôlant l'expression des facteurs de virulence

Mis à part le système *agr* et le facteur SigB, le génome de *S. aureus* contient potentiellement de nombreux autres systèmes participant à la régulation des facteurs de virulence (Novick, 2003). Plusieurs systèmes de transduction à deux composants dont les systèmes *sae* (Giraudo *et al.*, 1999; Giraudo *et al.*, 1994), *srr* (Throup *et al.*, 2001; Yarwood *et al.*, 2001) et *arl* (Fournier et Hooper, 2000) interviennent dans la régulation de l'expression des facteurs de virulence en réponse à des stimuli environnementaux. Le facteur de transcription SarA et ses homologues (*p. ex.* SarS, SarT et Rot) affectent aussi une grande variété de gènes dont des facteurs de virulence (Cheung *et al.*, 2001; Cheung et Zhang, 2002; Dunman *et al.*, 2001; Manna et Cheung, 2001; McNamara *et al.*, 2000; Schmidt *et al.*, 2001; Tegmark *et al.*, 2000). Les études établissant globalement la contribution des différents régulateurs de la virulence à la pathogenèse de *S. aureus* lors d'infections pulmonaires FK sont rares. Cependant, il a été suggéré que *sae* est une régulateur clé de l'expression des facteurs de virulence de *S. aureus* lors d'infections pulmonaires chez les patients FK (Goerke et Wolz, 2010). Il a récemment été montré que l'expression de petits ARN régulateurs peut être liée à différents types de modes de vie (croissance *in vitro*, colonisation nasale, formation d'abcès et infections pulmonaires FK) chez des échantillons cliniques de *S. aureus* (Song *et al.*, 2012), supportant l'hypothèse que des réseaux de régulation génétique pourraient être activés d'une façon différentielle lors d'infections pulmonaires chez les patients FK.

2.3. Mécanismes et facteurs de virulence potentiellement impliqués dans les infections chroniques causées par *S. aureus*

2.3.1. Adhésion tissulaire et formation de biofilm

Les biofilms sont des communautés microbiennes sessiles caractérisés par des cellules attachées à un substrat et entre elles et qui sont enchâssées dans une matrice de substances polymériques extracellulaires (Donlan et Costerton, 2002). Bien qu'un biofilm puisse être généré à partir d'une seule bactérie, les différentes conditions présentes à travers la communauté d'un biofilm semblent entraîner le développement de sous-populations distinctes. Ainsi, jusqu'à 4 états métaboliques différents peuvent être identifiés à l'intérieur d'un biofilm généré *in vitro* à partir de staphylocoques (Rani *et al.*, 2007). Bien que la formation de biofilm ne soit pas un prérequis aux infections persistantes (Kristian *et al.*, 2004), les biofilms ont la réputation d'être difficiles à éradiquer. Les biofilms semblent en effet conférer plusieurs avantages aux bactéries en comparaison au mode de croissance planctonique. La matrice extracellulaire des biofilms a la capacité de séquestrer et de concentrer certains nutriments en provenance de l'environnement (Beveridge *et al.*, 1997). Le biofilm permettrait aussi aux bactéries d'éviter certains mécanismes de défense de l'hôte ainsi que l'action des antibiotiques (De Beer *et al.*, 1994; Lewis, 2010; Singh *et al.*, 2010b; Xu *et al.*, 2000). Aussi, les biofilms ont la capacité d'amorcer des processus de dispersion par l'utilisation de réponses génétiquement programmées (Boles et Horswill, 2008; Boyd et Chakrabarty, 1994).

Le développement d'un biofilm est un processus comportant deux étapes physiologiquement bien distinctes : l'adhésion initiale à un substrat et la maturation subséquente vers une structure à l'architecture complexe. Dans l'hôte, l'adhésion des bactéries à la matrice extracellulaire représente donc la première étape de la formation d'un biofilm. Cet adhésion

initiale implique vraisemblablement les douzaines de protéines de *S. aureus* qui lient des composantes de la matrice extracellulaire de l'hôte et sont regroupées sous le terme MSCRAMM pour *microbial surface components recognizing adhesive matrix molecules* (Patti et Hook, 1994). Tandis que différentes composantes de l'hôte (tel que la fibronectine, le collagène, le fibrinogène et bien d'autres) sont liées par les MSCRAMM, de la redondance fonctionnelle est aussi observée puisqu'une même composante de l'hôte peut être liée par plusieurs MSCRAMM distinctes (Hauck et Ohlsen, 2006). La phase de maturation du biofilm est quant à elle caractérisée par l'agrégation intercellulaire et par l'utilisation de forces structurantes qui mèneront à l'apparence tridimensionnelle typique des biofilms matures, *c.-à-d.* des tours en forme de champignons entourées de canaux remplis de fluides. Les différents processus permettant le détachement du biofilm pourront alors être activés et assurés la dissémination du pathogène vers d'autres sites de l'organisme hôte (Otto, 2008).

Plusieurs mécanismes de formation de biofilm semblent exister chez *S. aureus* (Archer *et al.*, 2011). Le biofilm dépendant de PIA (*polysaccharide intercellular antigen*) est produit *in vitro* à partir de l'UDP-*N*-acétylglucosamine par l'action des produits du locus *ica* (*intercellular adhesion*) (Cramton *et al.*, 1999). Le locus *ica* a été démontré comme étant important à la formation du biofilm et à la virulence, et est exprimé sous des conditions particulières comme les environnements anaérobiques (Cramton *et al.*, 2001; Fitzpatrick *et al.*, 2005). Cependant, la formation de biofilm peut aussi avoir lieu d'une façon indépendante du locus *ica* (Fitzpatrick *et al.*, 2005; O'Neill *et al.*, 2007; Pozzi *et al.*, 2012; Toledo-Arana *et al.*, 2005). Plusieurs études suggèrent qu'une adhésion intercellulaire impliquant des facteurs protéiques peut remplacer PIA lors de la formation d'un biofilm. Ainsi, des résultats ont montré que la protéine A est essentielle à la formation d'un biofilm chez un mutant *ica* (Merino *et al.*, 2009). Les FnBPs (*fibronectin-binding proteins*) semblent aussi promouvoir la formation de biofilm par un mécanisme influencé par SigB et impliquant l'autolysine Atl (Houston *et al.*, 2011). Chez d'autres souches, la protéine Bap (*biofilm-associated protein*) et ses homologues peuvent conférer à la bactérie la capacité de s'agréger et de former du biofilm d'une façon

indépendante de PIA (Lasa et Penades, 2006). L'ADN extracellulaire semble un autre aspect important des biofilms et *S. aureus* utilise un certains nombre de mécanismes afin d'ajouter de l'ADN à la matrice de ses biofilms tels que le contrôle de l'activité de différentes hydrolases extracellulaires, l'induction de la mort cellulaire programmée et la lyse cellulaire (Brady *et al.*, 2006; Mann *et al.*, 2009; Resch *et al.*, 2005; Rice *et al.*, 2007).

Les régulateurs globaux influencent le développement du biofilm à plusieurs étapes. Le système *agr* influence la formation du biofilm en réprimant les facteurs d'adhésion associés à la surface bactérienne (Chan *et al.*, 2004) et promeut l'initiation des mécanismes de dispersion en influençant l'expression de protéases, de nucléases et de peptides aux activités détergentes (Beenken *et al.*, 2010; Boles et Horswill, 2008; Kong *et al.*, 2006; Lauderdale *et al.*, 2009). SigB contrôle aussi la formation du biofilm de différentes façons. L'expression de plusieurs facteurs impliqués dans les phases précoces du développement du biofilm tels que des facteurs d'agglutination, FnBPA et la coagulase est augmentée par l'activité SigB (Nair *et al.*, 2003; Nicholas *et al.*, 1999) tandis que celle de plusieurs facteurs associés à la dispersion des biofilms est réprimée (Kullik et Giachino, 1997). Des souches mutantes pour *sigB* ne peuvent pas former de biofilm et expriment activement RNAIII (Cassat *et al.*, 2006; Rachid *et al.*, 2000), et l'inactivation d'*agr* ou l'utilisation d'inhibiteurs de protéases permet à un mutant *sigB* de produire du biofilm (Cassat *et al.*, 2006; Lauderdale *et al.*, 2009). Cependant, il a aussi été suggéré que SigB pourrait réguler la formation de biofilm dépendant de PIA (Archer *et al.*, 2011; Jefferson *et al.*, 2003; O'Gara, 2007) et le rôle de SigB dans le développement du biofilm pourrait varier entre les souches (Valle *et al.*, 2003). Certaines études attribuent beaucoup d'importance au facteur de transcription SarA qui est exprimé dans les biofilms et influence leur formation en prévenant la dégradation de l'ADN et des protéines extracellulaires (Beenken *et al.*, 2003; Valle *et al.*, 2003). Le rôle potentiel de SigB et du système *agr* dans la régulation des différentes étapes du développement du biofilm de *S. aureus* est illustré à la Figure 4.

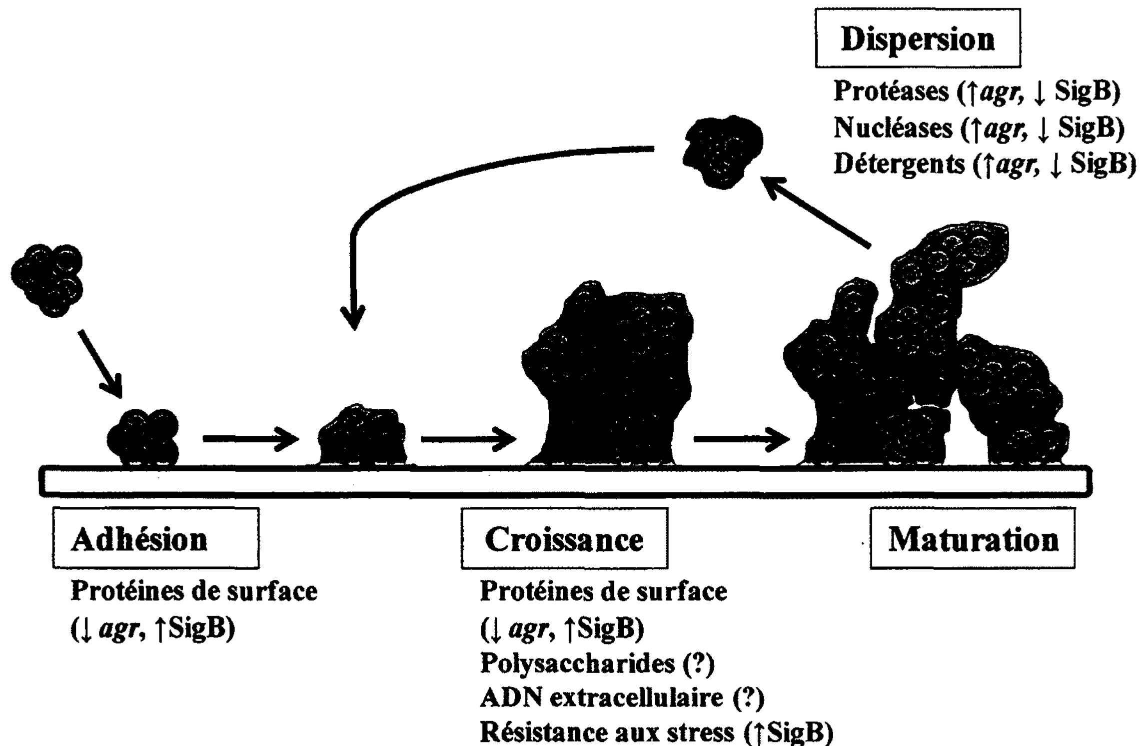

FIGURE 4. Rôle de SigB et du système *agr* dans la régulation de la formation du biofilm. SigB et le système *agr* interviennent dans les différentes étapes du développement du biofilm en régulant l'expression des protéines de surfaces, la production de certaines composantes de la matrice ainsi que celle d'agents de dispersion. Inspiré d'Archer *et al.* (2011) et d'Otto (2008).

2.3.2. Invasion et persistance dans les cellules de l'hôte

Bien que *S. aureus* soit maintenant considéré comme un pathogène intracellulaire facultatif (Garzoni et Kelley, 2009; Sendi et Proctor, 2009; Sinha et Fraunholz, 2010), le rôle précis de la persistance intracellulaire de *S. aureus* lors d'infections fait encore l'objet de débats et est difficile à démontrer sans équivoque expérimentalement (Garzoni et Kelley, 2009). Cependant, l'hypothèse veut que la persistance intracellulaire de *S. aureus* soit une stratégie pour échapper aux phagocytes professionnels et aux antibiotiques se retrouvant dans le milieu

extracellulaire. De plus, la niche intracellulaire pourrait servir en tant que réservoir aux infections récurrentes ou contribuer à la chronicité de certaines infections (Garzoni et Kelley, 2009; Lowy, 1998; Sinha et Fraunholz, 2010).

Bien que l'adhésion de *S. aureus* aux structures de l'hôte soit un prérequis à la colonisation, certaines protéines de la surface bactérienne, tels les FnBPs et la protéine Eap, sont aussi impliquées dans l'invasion des cellules de l'hôte (Sinha et Herrmann, 2005). Le mécanisme d'invasion le mieux caractérisé est celui par lequel un pont de fibronectine est formé entre les FnBPs et l'intégrine $\alpha_5\beta_1$ (Sinha *et al.*, 1999). L'internalisation de la bactérie procède alors par un mécanisme dépendant de l'actine filamenteuse et il a été démontré que les FnBPs sont non seulement importantes pour l'adhésion, mais aussi pour l'activation du remodelage du cytosquelette de la cellule hôte via la signalisation couplée aux intégrines (Sinha et Herrmann, 2005). Cependant, des mécanismes indépendants des FnBPs semblent exister (Kintarak *et al.*, 2004; Weidenmaier *et al.*, 2005) et certains résultats ont montré que, du moins sous certaines conditions, l'internalisation du *S. aureus* est multifactorielle (Werbick *et al.*, 2007) et possible sans FnBPs (Brouillette *et al.*, 2003a).

Plusieurs études rapportent une survie intracellulaire prolongée du *S. aureus* autant dans des lignées de cellules phagocytaires que dans des phagocytes non professionnels (Garzoni et Kelley, 2009; Sinha et Fraunholz, 2010) et il a été montré que *S. aureus* a la capacité de se répliquer dans certains types cellulaires (Kahl *et al.*, 2000; Kubica *et al.*, 2008). Cependant, il est aussi possible que certaines lignées cellulaires soient capables de tuer *S. aureus* (Deitch *et al.*, 1995). Il semble que la destinée des bactéries à l'intérieur de la cellule hôte soit influencée par l'existence de plusieurs différents compartiments cellulaires accueillants *S. aureus* et que la distribution du *S. aureus* intracellulaire parmi ces différents compartiments varie selon la souche à l'étude (Gresham *et al.*, 2000; Kubica *et al.*, 2008). De plus, il a été suggéré que *S. aureus* a la capacité de s'échapper des compartiments phagolysosomales dans les cellules qui

ne sont pas des phagocytes professionnels (Bayles *et al.*, 1998), mais, d'une façon contradictoire, il a récemment été démontré que l'intégrité du phagosolysome est généralement maintenue suite à l'invasion cellulaire (Lam *et al.*, 2010). Tel qu'illustré à la Figure 5, plusieurs différents scénarios sont envisageables pour le *S. aureus* intracellulaire : la clairance par dégradation lysosomale ou par des mécanismes de l'immunité innée (tels les peptides antimicrobiens, l'autophagie ou l'induction de l'apoptose), la persistance dans certaines vacuoles intracellulaires, l'évasion du phagolysosome et la persistance à l'intérieur du cytosol. L'induction de la mort cellulaire ainsi que les mécanismes moléculaires sous-jacents semblent aussi varier selon la lignée cellulaire et la souche de *S. aureus* à l'étude (Baran *et al.*, 2001; Haslinger-Loffler *et al.*, 2005; Haslinger *et al.*, 2003; Krut *et al.*, 2003; Schroder *et al.*, 2006). Il a été suggéré que l'accès au cytosol est un prérequis à l'induction de la mort de la cellule hôte (Haslinger-Loffler *et al.*, 2005; Klein *et al.*, 2006; Sinha et Fraunholz, 2010).

Il a été démontré que l'activation du système *agr* précède la sortie de la bactérie du phagolysosome (Qazi *et al.*, 2001) et qu'un mutant *agr* est incapable de sortir de ce compartiment (Jarry *et al.*, 2008). Conséquemment, des mutants *agr* ainsi que des mutants *sigB* influencent l'induction de la mort cellulaire (Haslinger-Loffler *et al.*, 2005; Kubica *et al.*, 2008). Bien que certains résultats suggèrent que l'α-hémolysine (Hla) soit le facteur contrôlé par le système *agr* qui intervient dans la sortie du phagolysosome (Jarry *et al.*, 2008; Kubica *et al.*, 2008), il semble maintenant que cette toxine n'est pas suffisante à elle seule et que la sortie du phagolysosome soit un processus multifactoriel (Giese *et al.*, 2009). Une récente étude suggère plutôt qu'une activité synergique entre la δ-hémolysine (Hld) et la β-hémolysine (Hlb) permet la sortie de *S. aureus* du phagolysosome chez des cellules épithéliales et endothéliales (Giese *et al.*, 2011). Des études génétiques plus complètes examinant les facteurs contribuant à la survie intracellulaire du *S. aureus* ainsi qu'à la réponse de la cellule hôte semblent requises.

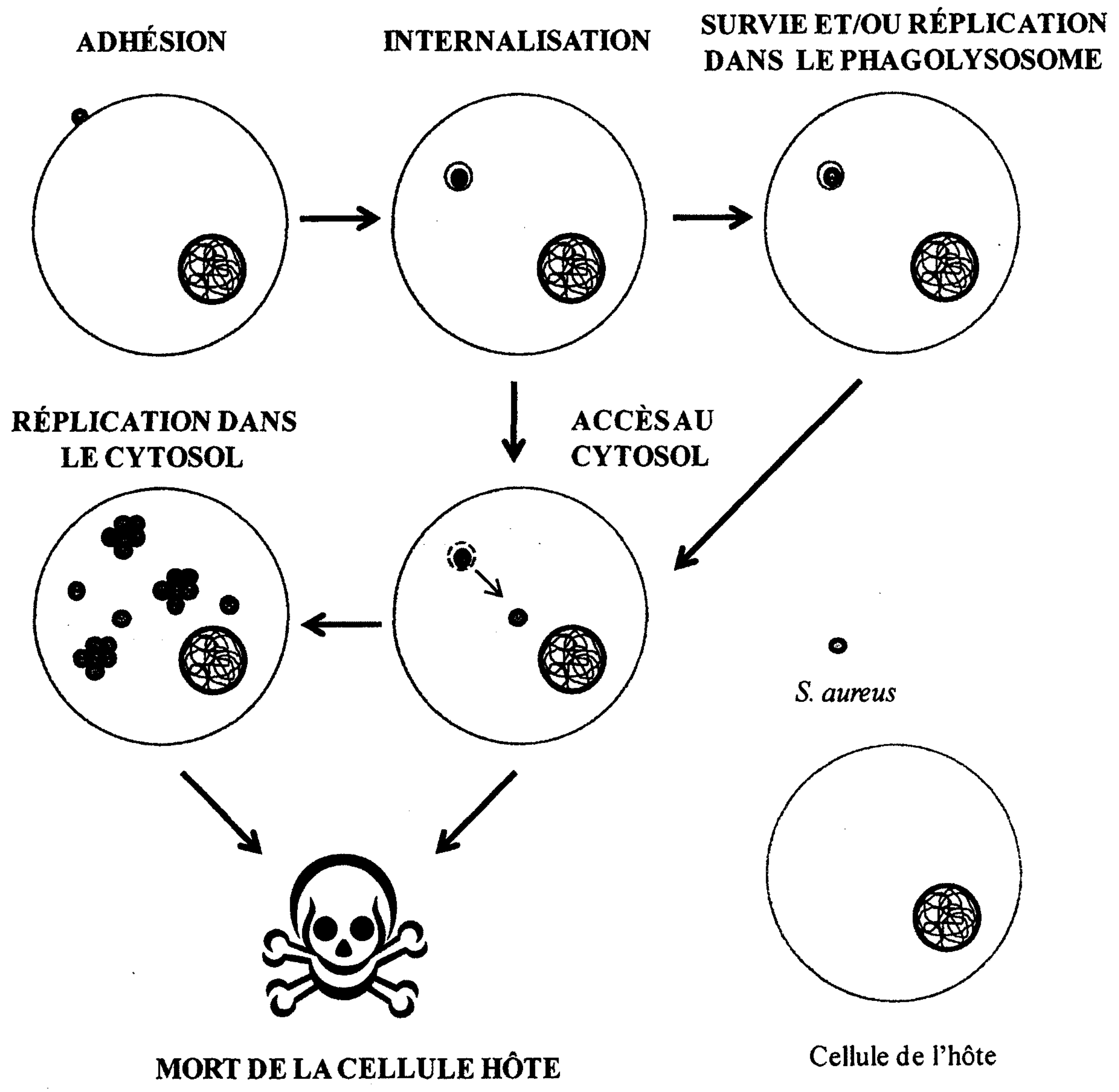

FIGURE 5. Destinées possibles du *S. aureus* après son internalisation par des phagocytes non professionnels. Inspiré de Sinha et Fraunholz (2010).

2.3.3. Modulation de la réponse immunitaire de l'hôte

S. aureus est bien équipé pour contrer les mécanismes de l'immunité innée et acquise de son hôte. Par diverses stratégies, cette bactérie a la capacité d'empêcher la migration des leucocytes, la fixation du complément, la reconnaissance des opsonines par les neutrophiles, l'induction efficace de la réponse à anticorps et de la mémoire immunitaire ainsi que de survivre à l'intérieur des phagocytes professionnels (Foster, 2005, 2009). De récents travaux montrent que la réponse immunitaire des cellules endothéliales peut grandement varier et est fortement dépendante des facteurs de virulence exprimés par les souches invasives de *S. aureus*. Plus spécifiquement, il est possible que la répression du système *agr* soit une stratégie adoptée par *S. aureus* pour se dissimuler à l'intérieur des cellules de l'hôte sans provoquer une réponse immunitaire (Grundmeier *et al.*, 2010). Ceci pourrait permettre à la bactérie d'échapper au système immunitaire et de causer des infections chroniques. Cependant, il a aussi été démontré que *S. aureus* est capable de survivre dans les macrophages pendant plusieurs jours et que cette survie requiert un système *agr* fonctionnel (Kubica *et al.*, 2008). Les macrophages pourraient alors servir de véhicules à la bactérie et ainsi promouvoir la dissémination des infections.

2.4. Bases moléculaires et contrôle de la virulence chez les SCVs de *S. aureus*

2.4.1. Manifestations cliniques et infections associées aux SCVs

Les variants à petites colonies ou SCVs (*small-colony variants*) sont des sous-populations de bactéries à la croissance lente présentant des caractéristiques phénotypiques bien distinctes de celles des souches prototypiques (voir Figure 6). Les SCVs se présentent sous la forme de

colonies à la morphologie atypique, produisent peu de pigmentation, d'hémolyse et de coagulases, sont moins susceptibles à certains antimicrobiens et ont des propriétés biochimiques inhabituelles. Des SCVs ont été décrits chez plusieurs différentes espèces bactériennes et isolés de plusieurs maladies. Ils reçoivent une attention particulière par la communauté scientifique, car il est possible qu'ils soient la cause d'infections persistantes et difficiles à traiter (Proctor *et al.*, 2006; Sendi et Proctor, 2009).

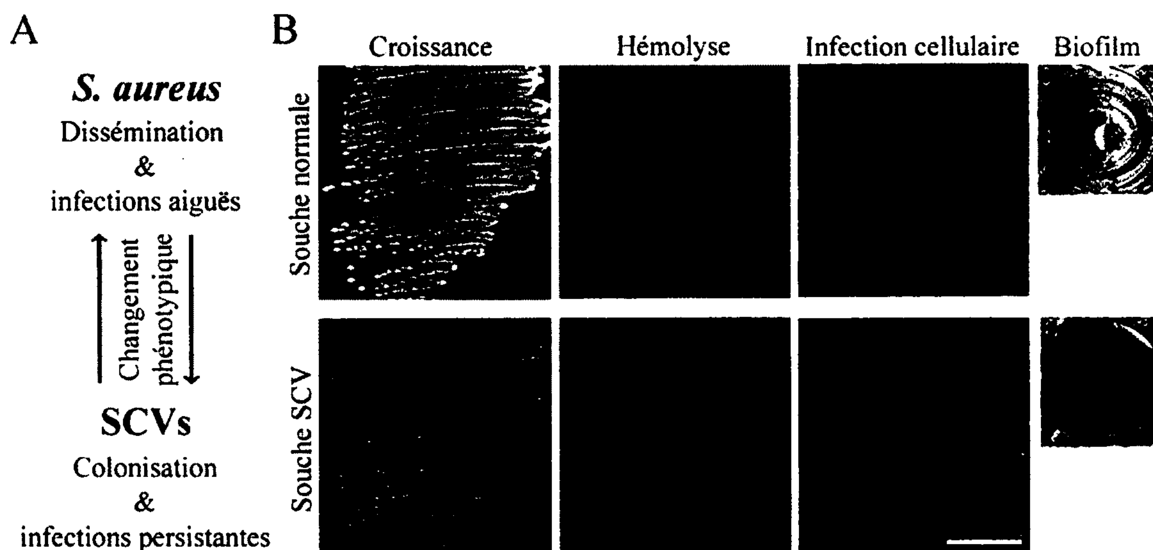

FIGURE 6. Comparaison de certaines caractéristiques phénotypiques entre les souches normales et SCVs. Tel que montré en A, *S. aureus* a l'habileté de pouvoir transiter entre le phénotype normal et SCV. Par comparaison avec les bactéries au phénotype normal, les SCVs ont un taux de croissance réduit, sont moins hémolytiques et ont une capacité accrue à infecter les cellules non phagocytaires et à former un biofilm (B). Les micrographies d'infection de cellules montrent l'actine de la cellule hôte en vert et les bactéries en rouge. La barre d'échelle représente 50 µm. Le biofilm a été révélé par une coloration au cristal violet. Modifié de Mitchell et Malouin (voir Annexe 1).

L'association entre les SCVs et les infections persistantes a plus particulièrement été observée pour *S. aureus* dans des cas d'ostéomyélites (von Eiff *et al.*, 1997a; von Eiff *et al.*, 2006b), de colonisation d'implants médicaux (Proctor *et al.*, 2006; Sanderson, 1999; Seifert *et al.*, 2003), d'arthrites septiques (Spearman *et al.*, 1996), d'infections pulmonaires chez les patients FK (Kahl *et al.*, 1998; Kahl *et al.*, 2003b) et de mammites bovines (Atalla *et al.*, 2008; Sompolinsky *et al.*, 1974). Du moins chez *S. aureus*, les SCVs ne sont pas rares, mais sont difficiles à récupérer des échantillons cliniques à cause de leurs exigences de croissance fastidieuses (Kipp *et al.*, 2005). Une incidence entre 1% et plus de 30% a été rapportée selon les études (Acar *et al.*, 1978; Kahl *et al.*, 2003b; von Eiff *et al.*, 1997a). Les SCVs sont particulièrement fréquents chez les patients FK, non seulement chez *S. aureus* (Kahl *et al.*, 2003b), mais aussi chez *P. aeruginosa* (Haussler *et al.*, 1999) et *B. cepacia* (Haussler *et al.*, 2003), et pourraient représenter une adaptation optimisée à la survie dans le milieu hostile que représente les voies respiratoires FK.

2.4.2. Facteurs favorisant l'émergence du phénotype SCV

Les souches SCVs provenant des échantillons cliniques ont souvent un phénotype instable et reprennent rapidement un taux de croissance normal, ce qui rend ces souches difficilement utilisables afin d'effectuer des expériences en laboratoire. Conséquemment, plusieurs de nos connaissances actuelles sur les SCVs ont été obtenues à l'aide de mutants stables de la chaîne de transport des électrons (Brouillette *et al.*, 2004; von Eiff *et al.*, 1997b; von Eiff *et al.*, 2006a) et peu de données concernant la dynamique de formation des SCVs sont actuellement disponibles. Il semble que l'émergence de SCVs soit stimulée ou du moins favorisée par une exposition aux aminoglycosides (Hoffman *et al.*, 2006; Massey *et al.*, 2001; Schaaff *et al.*, 2003; von Eiff *et al.*, 1997a), une exposition à certains exoproduits de *P. aeruginosa* (Biswas *et al.*, 2009; Hoffman *et al.*, 2006), les peptides cationiques (Sadowska *et al.*, 2002), le milieu intracellulaire de l'hôte (Tuchscherr *et al.*, 2011; Vesga *et al.*, 1996) et les infections

prolongées (Tuchscherr *et al.*, 2011). Plusieurs questions demeurent ouvertes concernant les signaux et mécanismes impliqués dans l'émergence de SCVs. Le(s) mécanisme(s) de formation de SCVs pourrait reposer sur des évènements génétiques stochastiques tels que l'acquisition de mutations aléatoires, des mécanismes génétiques plus complexes tels que ceux intervenant dans la variation de phase et des mécanismes de régulation (Abu-Qatouseh *et al.*, 2010; Massey *et al.*, 2001; Proctor *et al.*, 2006; Schaaff *et al.*, 2003). De plus, les mécanismes génétiques et de régulation ne sont pas mutuellement exclusifs. Le rôle potentiel des régulateurs globaux (*p. ex. agr*, SigB, SarA, etc) dans la formation de SCVs de *S. aureus* ne semble pas avoir été étudié jusqu'à maintenant.

2.4.3. Auxotrophies et voies métaboliques associées aux SCVs

Bien que plusieurs altérations du métabolisme bactérien puissent mener à un ralentissement de la croissance, un nombre limité de défectuosités semblent retrouvées chez les SCVs. Ainsi, les souches cliniques au phénotype SCV peuvent généralement être catégorisées en deux groupes : les SCVs à la chaîne de transport des électrons déficiente et les SCVs avec une défectuosité dans la biosynthèse de la thymidine. Des SCVs avec d'autres types d'auxotrophies ou avec des auxotrophies inconnues sont occasionnellement isolés (Proctor *et al.*, 2006). Les SCVs dépendants de la chaîne de transport des électrons sont incapables de faire la biosynthèse de la ménadione ou de l'hémine et, conséquemment, leur taux de croissance peut être supplémenté par l'ajout de ces molécules au milieu de croissance. Il reste cependant difficile de définir précisément la ou les lésion(s) génétique(s) expliquant le phénotype SCV des isolats cliniques, mais des mutations dans le gène *menB* ont été trouvées chez des isolats cliniques et semblaient être la cause de leur phénotype (Lannergard *et al.*, 2008). Des mutations générées en laboratoire dans les gènes *menD*, *hemB* et *cta* conduisent aussi au phénotype SCV (Bates *et al.*, 2003; Clements *et al.*, 1999; von Eiff *et al.*, 2006a). Comme illustré à la Figure 7, la défectuosité de la chaîne de transport des électrons de ce type

de SCVs explique plusieurs de leurs caractéristiques phénotypiques de par les effets attendus d'un flux énergétique diminué tel qu'un ralentissement de la croissance, une diminution du gradient électrochimique transmembranaire requis pour l'entrée massive des aminoglycosides dans la bactérie et une diminution de certains métabolismes secondaires ou accessoires telle que la production de pigments et de toxines (Proctor *et al.*, 2006).

Le phénotype des SCVs auxotrophes pour la thymidine est presque identique à celui des SCVs dépendants de la chaîne de transport des électrons, mais la raison pour cela n'est pas encore connue. Ce type de SCVs émerge à haute fréquence lors des traitements à long terme des patients FK avec la combinaison triméthoprime-sulfaméthoxazole (STX) (Gilligan *et al.*, 1987; Kahl *et al.*, 1998; Kahl *et al.*, 2003a). Le STX interfère avec la voie de synthèse de l'acide tétrahydrofolique, un cofacteur de la thymidylate synthase, et interfère avec la synthèse d'ADN. Il a été démontré qu'une souche clinique SCV auxotrophe pour la thymidine pouvait être complémentée par le gène *thyA* encodant justement la thymidylate synthase (Chatterjee *et al.*, 2008) et il est spéculé que les SCVs auxotrophes pour la thymidine contournent les voies inhibées par le STX en effectuant l'acquisition de thymidine extracellulaire. L'acquisition de thymidine extracellulaire se fait à l'aide de la pompe NupC chez *S. aureus* et est dépendante du gradient électrochimique (Saxild *et al.*, 1996; Smith *et al.*, 2005), soulignant un premier lien entre la chaîne de transport des électrons et les SCVs auxotrophes pour la thymidine, qui requiert de la ménaquinone au cours de sa biosynthèse (Proctor *et al.*, 2006). Bien que le phénotype de cette classe de SCVs soit directement lié à la quantité de thymidine se trouvant sans l'environnement, il est spéculé qu'une deuxième mutation dans *nupC* se retrouve dans leur génome et ralentit l'acquisition de thymidine extracellulaire (Proctor *et al.*, 2006).

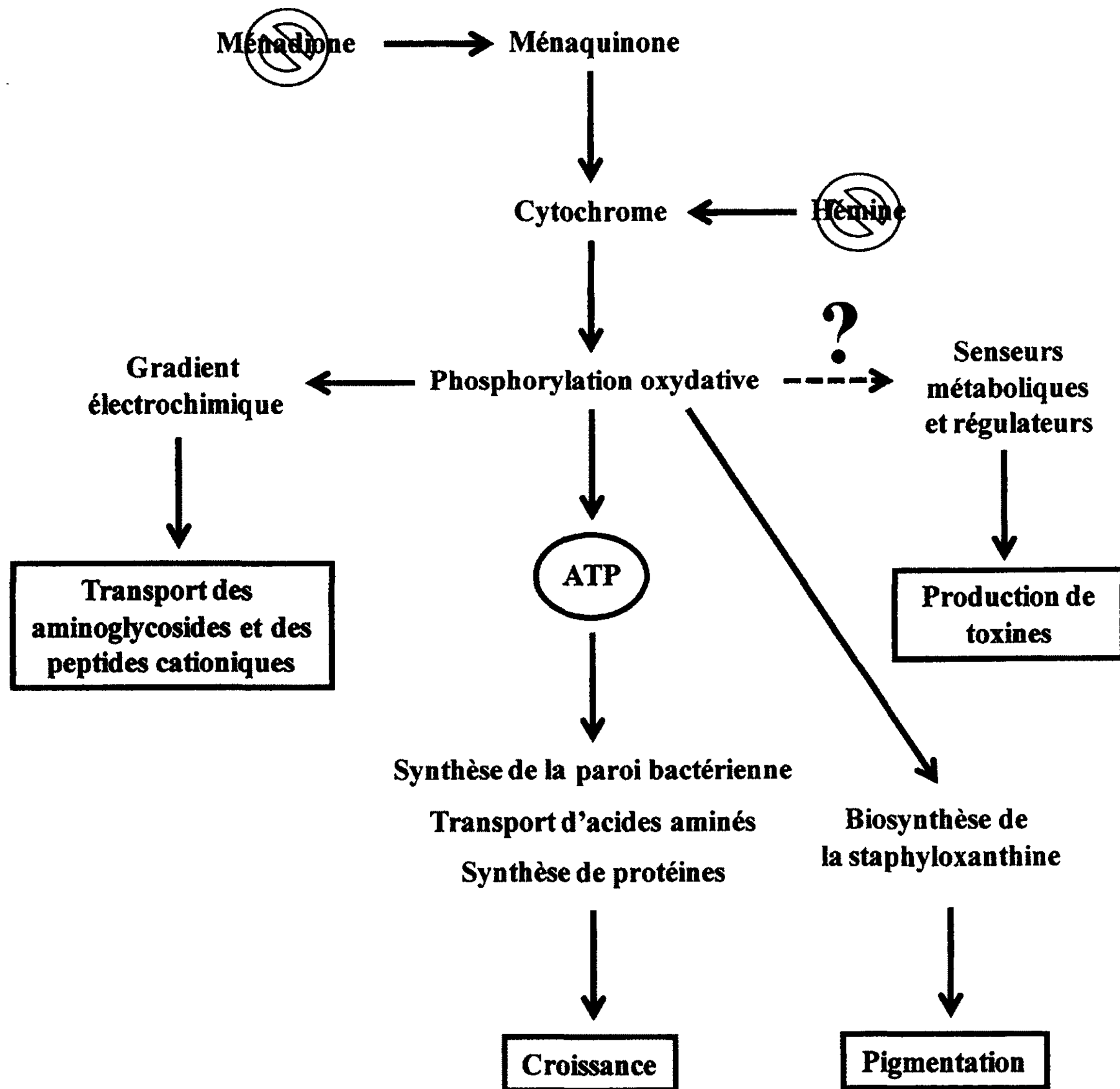

FIGURE 7. Association entre la chaîne de transport des électrons et le phénotype SCV.
Le point d'interrogation souligne que les détecteurs métaboliques et les régulateurs liant le niveau énergétique de la bactérie et la production de toxines sont encore inconnus chez *S. aureus*. Inspiré de Proctor *et al.* (2006).

2.4.4. Contrôle de l'expression des facteurs de virulence chez les SCVs

La production des facteurs de virulence est altérée chez les SCVs par rapport à celle des souches à croissance rapide. Concordant très bien avec leur faible activité hémolytique, il a été démontré que les SCVs produisent de très faibles quantités de Hla (Kohler *et al.*, 2003; von Eiff *et al.*, 1997b). Il semble toutefois que l'expression d'adhésines tels ClfA et les FnBPs soit augmentée chez ces variants, ce qui est corroboré par leur plus grande capacité à lier certaines molécules de la matrice extracellulaire de l'hôte (Vaudaux *et al.*, 2002). Ce patron particulier d'expression des facteurs de virulence semble explicable par une activité anormale de certains régulateurs globaux (Kahl *et al.*, 2005) ou l'activité SigB est constitutive et celle du système *agr* est faible (Moisan *et al.*, 2006; Senn *et al.*, 2005a). Bien que l'expression de *sarA* est accrue chez les SCVs (Moisan *et al.*, 2006), ce gène n'est probablement pas un des facteurs dominants contrôlant la virulence des SCVs étant donné que sa transcription est sous l'influence de SigB (Bischoff *et al.*, 2004; Deora *et al.*, 1997). Pour l'instant, il est difficile d'exclure l'idée que d'autres facteurs interviennent dans la régulation de l'expression des facteurs de virulence chez les SCVs. Ainsi, une récente étude montre que de petits ARN non-codants pourraient participer au phénotype SCV (Abu-Qatouseh *et al.*, 2010). Il fut toutefois suggéré que l'activation altérée de SigB et d'*agr* des SCVs explique le profil d'expression de plusieurs gènes encodant des protéines de surface et des exoprotéines (Moisan *et al.*, 2006). Cependant, l'impact de l'inactivation génétique de *sigB* sur l'expression des facteurs de virulence n'a pas encore été évalué dans une souche SCV.

2.4.5. Pathogenèse des infections aux SCVs

La caractéristique des SCVs la plus souvent évoquée afin d'expliquer l'importance de ces variants lors d'infections persistantes est leur habileté à entrer et à persister à l'intérieur des

phagocytes non professionnels (*p. ex.* les cellules épithéliales et les cellules endothéliales) (Sendi et Proctor, 2009). À cause de l'expression accrue d'adhésines (*p. ex.* ClfA et FnBPs) (Vaudaux *et al.*, 2002), les SCVs s'attachent efficacement aux cellules de l'hôte et activent probablement à un niveau supérieur la signalisation cellulaire subséquente à l'engagement des intégrines menant à la réorganisation du cytosquelette et à l'internalisation des bactéries (Clements *et al.*, 1999; Haslinger-Loffler *et al.*, 2005; Vaudaux *et al.*, 2002; Wang *et al.*, 2006). Une fois internalisés, il est suggéré que les SCVs facilitent leur survie intracellulaire en évitant d'endommager la cellule hôte, ce qui est possiblement attribuable au faible niveau d'activation du système *agr* (Moisan *et al.*, 2006). Le rôle du système *agr* et de celui d'autres régulateurs de la virulence lors de l'interaction des SCVs avec la cellule hôte demeure toutefois à définir.

Les SCVs pourraient être particulièrement bien équipés pour résister aux mécanismes de défense intracellulaire de la cellule hôte. L'activité SigB des SCVs mène à l'expression de gènes impliqués dans la réponse bactérienne aux stress (Moisan *et al.*, 2006) et permet de résister à plusieurs stress environnementaux (Chan *et al.*, 1998; Gertz *et al.*, 1999; Horsburgh *et al.*, 2002). De plus, il a été montré que leur paroi cellulaire est particulièrement épaisse (Bulger et Bulger, 1967) et une souche SCVs de *S. epidermidis* surproduisait le polysaccharide PIA (Al Laham *et al.*, 2007), reconnue pour contribuer à la résistance à l'activité bactéricide rencontrée à l'intérieur des neutrophiles (Ulrich *et al.*, 2007). Le potentiel de membrane altéré des SCVs (Baumert *et al.*, 2002; Koo *et al.*, 1996) leur confère possiblement une résistance accrue aux peptides antimicrobiens cationiques (Peschel, 2002) qui sont produits par les macrophages, les neutrophiles et les cellules épithéliales (Lehrer et Ganz, 1999). Tandis qu'une étude suggère que les SCVs sont capables de s'échapper du phagolysosome (Balwit *et al.*, 1994), une autre montre que les SCVs résistent mieux que leur contrepartie à la croissance normale à l'activité bactéricide des phagolysosomes des cellules endothéliales (Schroder *et al.*, 2006). Finalement, probablement à cause de la faible activité de leur système *agr* (Grundmeier *et al.*, 2010), les SCVs semblent capables de persister dans les cellules de l'hôte en évitant

considérablement l'activation du système immunitaire (Tuchscherr *et al.*, 2010; Tuchscherr *et al.*, 2011). Cependant, de récents travaux suggérant que FnBPA est impliqué dans l'induction de NF-κB chez des cellules non phagocytaires viennent brouiller les conclusions (Shinji *et al.*, 2011). Toutefois, l'hypothèse d'une faible stimulation du système immunitaire par les SCVs est supportée par des cas cliniques où des infections aux SCVs persistaient d'une façon asymptomatique pendant plusieurs années (Kipp *et al.*, 2003; Proctor *et al.*, 1995).

Les SCVs semblent être de bons producteurs de biofilm (Al Laham *et al.*, 2007; Haussler, 2004; Singh *et al.*, 2009, 2010a; von Gotz *et al.*, 2004). Bien que certains résultats suggèrent que les SCVs des staphylocoques forment du biofilm par un mécanisme dépendant de la production de PIA (Al Laham *et al.*, 2007; Singh *et al.*, 2010a), le niveau d'expression des protéines de surface ainsi que la faible activité du système *agr* semblent plutôt suggérer la formation d'un biofilm dépendant des protéines extracellulaires. L'habileté des SCVs à former un biofilm a été une caractéristique relativement peu étudiée et qui pourrait jouer un très grand rôle dans la propension de ces souches à causer des infections chroniques. La caractérisation des mécanismes moléculaires impliqués dans la formation de biofilms des SCVs pourrait donc révéler de nouvelles cibles thérapeutiques.

Dans les modèles expérimentaux d'infections animales, les SCVs ont la capacité de causer des infections et peuvent être réisolés en cultures pures. Dans plusieurs modèles, les SCVs sont moins virulents, mais tout de même capables de persister (Proctor *et al.*, 2006). Dans un modèle murin d'arthrite septique, une souche SCV causait la maladie à une plus haute fréquence et avec une plus grande sévérité qu'une souche normale (Jonsson *et al.*, 2003). Il a aussi été observé qu'une souche SCV persistait mieux que sa contrepartie à la croissance normale en présence de céphapirine dans un modèle de mammite murine (Brouillette *et al.*, 2004). D'une façon semblable, bien qu'une thérapie à l'oxacilline dans un modèle d'endocardite réduisait l'infection de végétations cardiaques par les SCVs, aucun effet n'était

observé dans le rein et dans la rate pour une souche SCV en comparaison à une souche normale (Bates *et al.*, 2003). Il a récemment été montré que le phénotype SCV émerge d'infections prolongées et que ce phénotype pourrait constituer une stratégie pour échapper au système immunitaire de l'hôte et établir une infection chronique (Tuchscherr *et al.*, 2011). Cependant, l'importance de différents facteurs ou régulateurs de virulence dans la pathogenèse des SCVs ne semble pas avoir été étudiée dans les modèles d'infections expérimentales. De plus, il n'a jamais été montré que le phénotype SCV confère un avantage lors d'infections pulmonaires expérimentales.

OBJECTIFS ET HYPOTHÈSES DE DOCTORAT

Mon objectif général de doctorat a été d'étudier les fondements moléculaires de la persistance de *S. aureus* lors d'infections pulmonaires chroniques chez les patients FK.

Deux hypothèses ont été centrales à mes recherches :

- Le phénotype SCV confère un avantage à *S. aureus* lors d'infections chroniques des voies respiratoires FK.
- La virulence des SCVs de *S. aureus* est contrôlée ou, du moins, influencée par l'activité du facteur de transcription sigma alternatif sigma B.

Voici mes principaux objectifs spécifiques :

- Caractériser les mécanismes moléculaires qui influencent la virulence des SCVs (voir Chapitres 1, 2, 3, 4, 5 et Annexe 3).
- Déterminer la contribution de certains facteurs environnementaux et génétiques à l'émergence de SCVs, la formation de biofilm ou la persistance intracellulaire de *S. aureus* dans un contexte FK (voir Chapitres 2, 3 et 4).
- Évaluer la tomatidine dans le contexte du développement d'antibiothérapies alternatives permettant de combattre les infections pulmonaires chroniques à *S. aureus* chez les patients FK (voir Chapitres 6 et 7).

CHAPITRE 1

Staphylococcus aureus SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients

1.1. Introduction de l'article

L'objectif de cette étude a été de caractériser l'interaction entre la fibronectine et des SCVs isolés de patients FK ainsi que d'évaluer l'influence de SigB sur la fréquence de cette interaction. En collaboration avec l'étudiant Charles-Antoine Carpin Lamontagne et Gilles Grondin, une technique de spectroscopie de force atomique permettant de mesurer l'interaction fibronectine-*S. aureus* a été développée dans le laboratoire du Pr Michel Grandbois. L'étudiant Réjean Lebel (labo Grandbois) a aussi participé indirectement au projet par son expertise en programmation informatique (voir Annexe 2, Résumé 6). Le laboratoire du Pr Brian G. Talbot a fait la synthèse d'un peptide recombinant et Éric Brouillette a participé à la génération de mutants. J'ai participé à la mise au point du protocole de spectroscopie de force atomique et d'analyse des données. J'ai effectué les expériences de spectroscopie de force atomique, de PCR quantitative en temps réel et participé à la génération d'un mutant. J'ai rédigé l'ébauche de l'article, effectué la conception des figures et participé à la correction du manuscrit jusqu'à sa publication.

Référence de l'article :

Mitchell, G., Lamontagne, C.A., Brouillette, E., Grondin, G., Talbot, B.G., Grandbois, M., and Malouin, F. (2008). *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. Mol. Microbiol. *70*, 1540-1555.

1.2. Résumé en français de l'article

Les gènes encodant des protéines de surface et régulés par SigB sont exprimés de façon constitutive chez les SCVs de *Staphylococcus aureus* isolés des patients FK. Notre hypothèse est que ces SCVs sont verrouillés dans un état de colonisation en maintenant l'expression d'adhésines telles les protéines liant la fibronectine (FnBPs) pendant leur croissance. La spectroscopie de force a été utilisée afin d'étudier l'interaction fibronectine-FnBPs parmi des souches dont l'activité SigB diffère. L'interaction fibronectine-FnBPs a été décrite par une force de 1000+/-400 pN (vitesse de rétraction de 2 µm/s), une barrière énergétique d'une largeur de 0.6+/-0.1 Å et un taux de dissociation de 2 x 10^{-4} s^{-1}. Un isolat SCV FK exprimait *fnbA* d'une façon accrue pendant toutes les phases de croissance et démontrait une capacité soutenue à lier la fibronectine tandis qu'une souche prototypique liait la fibronectine à une fréquence réduite durant la phase stationnaire lorsque l'expression de *fnbA* était réprimée. Une expression réduite de *fnbA* était observée chez les mutants *sigB*, ce qui était associé à une diminution de l'adhésion à la fibronectine. Ces résultats suggèrent que l'interaction fibronectine-FnBPs joue un rôle dans la formation d'une adhésion mécaniquement résistante de *S. aureus* aux tissus de l'hôte et supportent l'hypothèse que les SCVs isolés de la FK sont verrouillés dans un état de colonisation par une activité constitutive de SigB.

1.3. L'article scientifique

Staphylococcus aureus **SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from Cystic Fibrosis Patients**

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Running title: SigB Activity and the Fibronectin-*S. aureus* Interaction.

Keywords: SCV; FnBP; Host-Pathogen interaction; Cystic fibrosis, Atomic force microscopy.

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SUMMARY

Genes encoding cell-surface proteins regulated by SigB are stably expressed in *Staphylococcus aureus* small-colony variants (SCVs) isolated from cystic fibrosis (CF) patients. Our hypothesis is that CF-isolated SCVs are locked into a colonization state by sustaining the expression of adhesins such as fibronectin-binding proteins (FnBPs) throughout growth. Force spectroscopy was used to study the fibronectin-FnBPs interaction among strains varying for their SigB activity. The fibronectin-FnBPs interaction was described by a strength of 1000 ± 400 pN (pulling rate of 2 μm/s), an energetic barrier width of 0.6 ± 0.1 Å and an off-rate below 2×10^{-4} s^{-1}. A CF-isolated SCV highly expressed *fnbA* throughout growth and showed a sustained capacity to bind fibronectin, whereas a prototypic strain showed a reduced frequency of fibronectin-binding during the stationary growth phase when its *fnbA* gene was down-regulated. Reduced expression of *fnbA* was observed in *sigB* mutants, which was associated with an overall decrease adhesion to fibronectin. These results suggest that the fibronectin-FnBPs interaction plays a role in the formation of a mechanically resistant adhesion of *S. aureus* to host tissues and supports the hypothesis that CF-isolated SCVs are locked into a colonization state as a result of a sustained SigB activity.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen causing various nosocomial and community-acquired infections, ranging from superficial skin lesions to life-threatening diseases (Lowy, 1998). The broad spectrum of diseases caused by *S. aureus* is related to its numerous virulence factors which affect host defenses, allow host colonization, cause destruction of tissues and induce sepsis syndromes (Archer, 1998). The expression of these virulence factors is under the control of global regulators such as Agr and SigB. It is thought that the Agr system controls the transition from the colonization to the invasion phase (Novick, 2003), whereas the alternative transcription factor SigB is involved in stress responses and positively influences adhesin expression when needed for host colonization (Nair *et al.*, 2003;Bischoff *et al.*, 2004; Entenza *et al.*, 2005).

S. aureus infections are often persistent and a single strain can infect a host for several years. In numerous chronic infections, *S. aureus* isolates were often found to exhibit a dysfunctional oxidative metabolism (Proctor *et al.*, 1994). This defect caused an alteration in the expression of virulence factors, a slower growth and a loss of colony pigmentation. These *S. aureus* variants were named Small-Colony Variants (SCVs) because of the small-size of the colonies when streaked on agar (Looney, 2000). SCVs are frequently isolated from cystic fibrosis (CF) patients with chronic infections of the airways (Kahl *et al.*, 1998; Sadowska *et al.*, 2002), as well as from other chronic infections such as osteomyelitis (von Eiff *et al.*, 2006), septic arthritis (Spearman *et al.*, 1996), bovine mastitis (Sompolinsky *et al.*, 1974; Atalla *et al.*, 2008) and infections of orthopedic devices (Sanderson, 1999). Various studies showed that SCVs possess an increased capacity to adhere, invade and persist in host cells (Kahl *et al.*, 1998; Kahl *et al.*, 2003; Brouillette *et al.*, 2004; Moisan *et al.*, 2006). In turn, this ability to survive within host cells may help the establishment of chronic infections by offering the

bacteria a protection from the immune system and the antibiotic action (Alexander and Hudson, 2001; Brouillette *et al.*, 2004).

We have recently demonstrated that the transcription of several virulence factors in *S. aureus* SCVs isolated from CF patients is strongly influenced by SigB. We have also shown that SigB activity is associated with an increased capacity of the bacterium to invade and persist within cells. Importantly, several genes encoding a variety of known adhesins and cell surface proteins were actively expressed in these clinically isolated SCVs (Moisan *et al.*, 2006). This agrees with the generally accepted concept that cellular invasion of mammalian cells by *S. aureus* involves the expression of adhesins such as fibronectin-binding proteins or FnBPs (Dziewanowska *et al.*, 1999; Vaudaux *et al.*, 2002).

The FnBPs bind fibronectin, a modular glycoprotein found in the extracellular matrix and on the surface of a variety of cells (Pankov & Yamada, 2002). The principal *S. aureus* FnBPs, FnBPA and FnBPB, have similar structures and share many characteristics with fibronectin-binding adhesins of other staphylococci and streptococci (Joh *et al.*, 1994). The interaction between fibronectin and *S. aureus* FnBPs is highly multivalent and is mediated by several high affinity binding sites (Shwarz-Linek *et al.*, 2003; Meenan *et al.*, 2007). It has been suggested that the resulting bond might be irreversible under physiological conditions (Proctor *et al.*, 1982). Reddy and Ross (2001) have shown that FnBPs are involved in the adherence of *S. aureus* to bovine aortic endothelial cells under conditions of flow that mimic the physiological shear stress found in blood vessels. Moreover, we have already shown that the presence of FnBPs on *S. aureus* increased the bacteria's capacity to colonize mammary glands under suckling pressure in a mouse model of mastitis (Brouillette *et al.*, 2003). Such studies suggest that the fibronectin-FnBPs interaction mediates a mechanically resistant adhesion to host tissues.

Atomic force microscopy (AFM)-based force spectroscopy studies are increasingly being used in biology for the characterization of mechanisms involved in single molecular recognition pairs (for review see Zlatanova *et al.*, 2000; Lee *et al.*, 2007). Microbiologists can use this technique to characterize the strength of interactions involved in establishing the contact between a bacterium and the host, a process which often takes place under mechanical stresses (for review see Dufrêne, 2004). Furthermore, Evans & Ritchie (1997) have derived a dynamic force spectroscopy approach from Bell's model of cell to cell adhesion (Bell, 1978) that allows the study of molecular dissociation pathways under applied forces in order to reveal energetic barriers and force-mediated dissociation rates. The dissociation parameters of several intermolecular interactions under external forces have since been determined using this approach (for review see Lee *et al.*, 2007).

The main aim of this study was to evaluate the influence of SigB activity on the fibronectin-binding ability of *S. aureus* in prototype and cystic-fibrosis SCV backgrounds. Using AFM-based force spectroscopy, we have measured the strength, the compliance and have estimated the resistance of the fibronectin-FnBPs interaction on living bacteria. We have also examined the relation between the level of SigB activity and the fibronectin-binding ability of *S. aureus*. We show that the enhanced influence of SigB in a SCV isolated from a CF patient resulted in the up-regulated expression of *fnbA* throughout growth and thus led to a higher frequency of fibronectin-binding. We suggest that the ability of some *S. aureus* strains or variants to sustain elevated SigB activity may allow persistent colonization of host tissues.

RESULTS

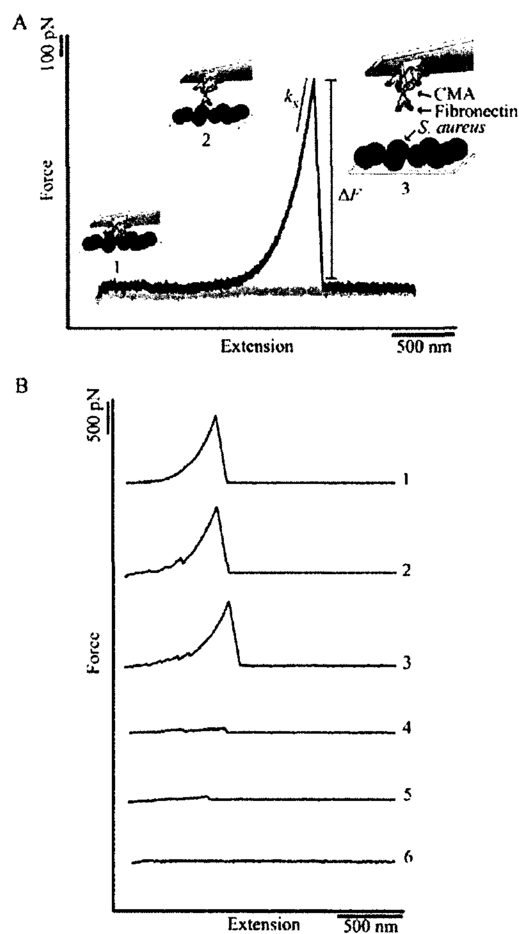

FIGURE 1. AFM-based force spectroscopy of the fibronectin-*S. aureus* interaction. (A) The experimental setup consists of fibronectin proteins and bacteria covalently linked, respectively, to an AFM probe and a glass slide. A carboxymethylamylose (CMA) spacer was used to tether the fibronectin to the probe whereas activated citrate was used to immobilize *S. aureus* to the glass slide. During a typical experiment, approach (grey line) and retraction (black line) curves showing rupture events were obtained in three steps: the tip was brought

into contact with the bacteria (1), the tip was retracted (2) and the interaction between the tip and the bacteria was ruptured (3). ΔF is the force needed to rupture this interaction and k_s is the apparent spring constant of the system derived from the slope of the rupture peak. (B) Typical retraction curves observed with the fibronectin decorated AFM probe and the strain Newbould in early exponential growth. The curves 1-3 show the rupture of strong interactions whereas the curves 4 and 5 show the rupture of relatively weak and non specific interactions. The curve 6 is typical for an approach-retract cycle producing no tip-sample interaction.

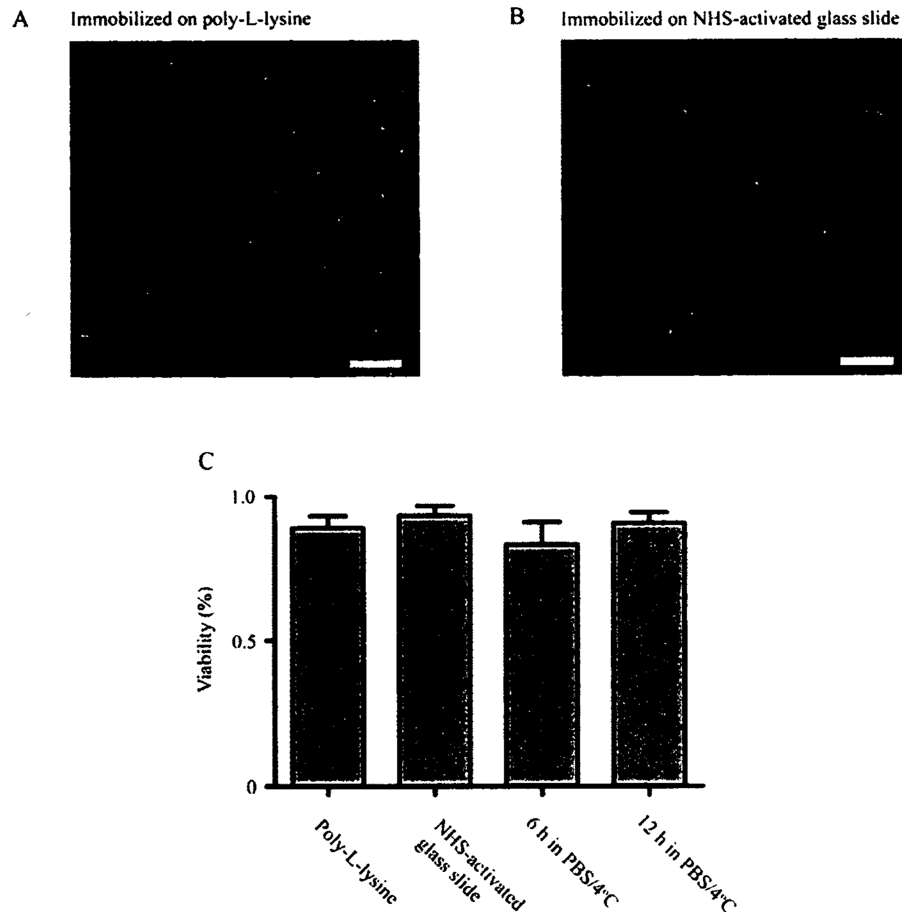

FIGURE S1. Viability of *S. aureus* following immobilization on NHS-activated glass slides. Fluorescence microscopy was used to evaluate the viability of *S. aureus* Newbould immobilized

on the activated glass slide; living bacteria are green and dead bacteria are red. The bacteria were grown to the early exponential phase prior to immobilization. A) Bacteria immobilized on poly-L-lysine Petri dish and B) Bacteria immobilized on NHS-activated glass slide. The scale bar corresponds to 10 μm. C) Quantification of the viability of bacteria immobilized on poly-L-lysine Petri dish and on NHS-activated glass slide after 0, 6 and 12 h of incubation in PBS at 4°C. Quantifications were done by counting between 30 and 300 bacteria in duplicate from two independent experiments. Results are expressed as means and standard deviations. Results for the tested conditions are not significantly different according to a one-way analysis of variance followed by the Tukey's test.

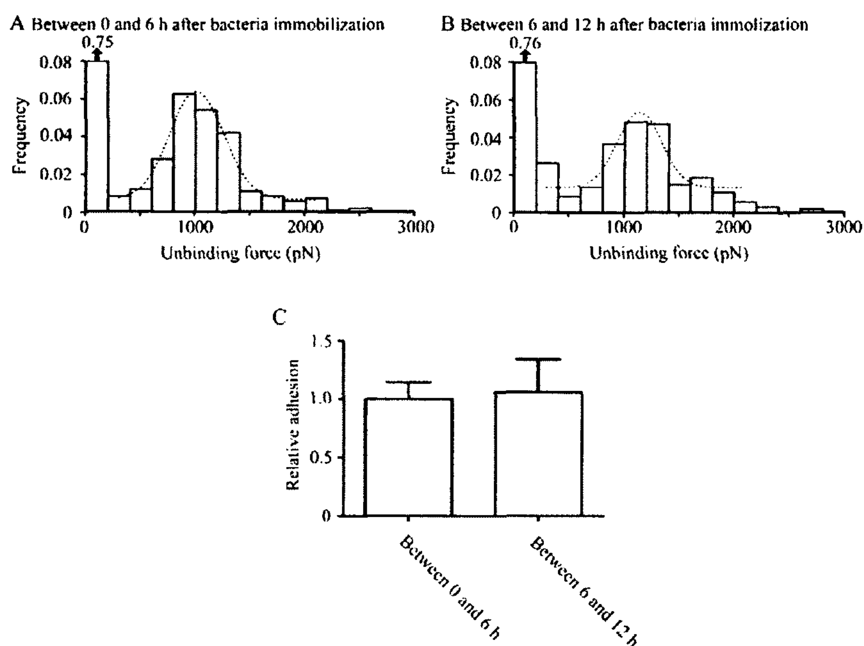

FIGURE S2. Effect of the incubation of bacteria for up to 12 h in PBS at 4°C on the fibronectin-FnBPs unbinding forces measured. Histograms present unbinding forces of the interaction between fibronectin and Newbould in the early exponential growth phase acquired between 0 and 6 h (n=805 from 4 independent experiments) (A) or between 6 and 12 h of

incubation in PBS at 4°C (n=781 from 5 independent experiments) (B). Numbers beside the arrows correspond to the total frequency of rupture events between 0 and 200 pN. Mean unbinding forces in (A) and (B) are 1100 ± 400 pN and 1200 ± 500 pN, respectively. Relative adhesions are also shown (C). Results have been normalized with the adhesion obtained for Newbould incubated between 0 and 6h in PBS at 4°C. Standard deviations are indicated. An unpaired *t*-test revealed no significant differences between the relative adhesions of both conditions.

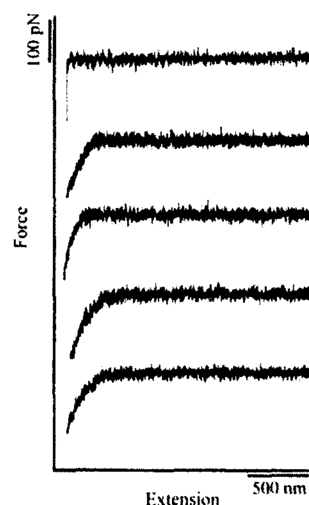

FIGURE S3. Localization of bacteria by using the difference in the elasticity of the surfaces contacted. Typical approach curves recorded on a glass slide and on bacteria are shown. The first curve is representative of the contact between a glass slide and the AFM probe whereas other curves are representative of the contact between a bacterium and the AFM probe. The presence of bacteria at positions contacted was confirmed according to the surface's elasticity evaluated from the retract curves.

AFM-based force spectroscopy of the fibronectin-*S. aureus* interaction. In order to evaluate the molecular strength of the fibronectin-*S. aureus* interaction and to explore the genetic regulation of the fibronectin-binding process on the surface of living bacteria, we used the experimental design depicted in Fig. 1A. This approach is based on a setup consisting of fibronectin proteins and bacteria covalently attached, respectively, to an AFM probe and to a glass slide. The viability of the bacteria on the glass slide was not altered by the cell immobilization technique (Fig. S1). Neither the viability of the bacteria (Fig. S1) nor the measured unbinding forces were altered during the experimental procedure (Fig. S2). During a typical experiment, the fibronectin-decorated probe is first positioned over the bacterial surface using a phase contrast light microscope conjugated to the AFM. The probe is then brought into contact with the bacteria and retracted. Forces associated to ruptures of specific molecular interactions between fibronectin and *S. aureus* were measured with a precision of a few piconewtons. The elasticity of the surface contacted by the probe was evaluated to confirm the presence of a bacterium at all positions studied and for each approach-retract cycle (Fig. S3) (for review on the investigation of microbial surface properties using AFM see Bolshakova *et al.*, 2004; Ubbink and Shär-Zammaretti, 2005). Typical fibronectin-*S. aureus* retraction curves are presented in Fig 1B. Both strong (curves 1-3) and weak unbinding forces (curves 4 and 5) were observed during the retraction of the fibronectin probe from the bacterial surface. Weak unbinding forces were considered as non-specific because they were observed at a similar frequency with an AFM tip functionalized only with the molecular spacer (data not shown). Retraction curves that demonstrated no tip-sample interaction were also generated (Fig 1B, curve 6).

The dissociation of fibronectin-*S. aureus* FnBPs complexes requires strong mechanical forces. Recorded forces of the fibronectin dissociation from the prototypic Newbould strain in early exponential growth displayed a group of measurements with a mean value of 1100 ± 400 pN (Fig. 2A) while the force distribution of the dissociation of CMA probes from Newbould did not show this grouping (Fig. 2B). This demonstrates that the generation of strong

interactions between the probe and bacteria is mediated through the fibronectin and not through the molecular spacer used to attach the protein to the probe. Competition experiments were carried out using fibronectin proteins added in solution to demonstrate the specific involvement of probe-bound fibronectin in the measured interaction with *S. aureus*. Fig. 2C shows that the presence of free fibronectin in the experimental buffer almost completely abolished the occurrence of fibronectin-*S. aureus* unbinding events.

FnBPA and FnBPB are considered as the main FnBPs of *S. aureus* (Greene *et al.*, 1995). To study their importance in the ability of the bacterium to bind fibronectin, we also conducted competition experiments with the fibronectin-binding region D1D2D3 of these FnBPs. It has been shown that the D1D2D3 fragment binds fibronectin and inhibits fibronectin-binding to *S. aureus* (McGavin *et al.*, 1993). The presence of the recombinant protein fragment in the experimental media caused a clear decrease in the frequency of high-force interactions (Fig. 2D). The implication of FnBPA and FnBPB in the interaction of *S. aureus* with the fibronectin decorated probe was further confirmed using strain 8325-4 and its isogenic *fnbAB* mutant, DU5883, in early exponential growth. The differential fibronectin-binding ability of these strains has already been characterized by others (Dziewanowska *et al.*, 2000; Reddy and Ross, 2001; Brouillette *et al.*, 2003). The unbinding forces histogram of the fibronectin dissociation from strain 8325-4 still showed a group of rupture forces with a mean value of 900 ± 500 pN (Fig. 2E) whereas this group was not observed with the *fnbAB* mutant DU5883 (Fig. 2F). Fig.2G and 2H present relative adhesions of the AFM probe to bacteria derived from these various experimental conditions. The statistical significance of our results demonstrates that we were indeed measuring the fibronectin-FnBPs interaction.

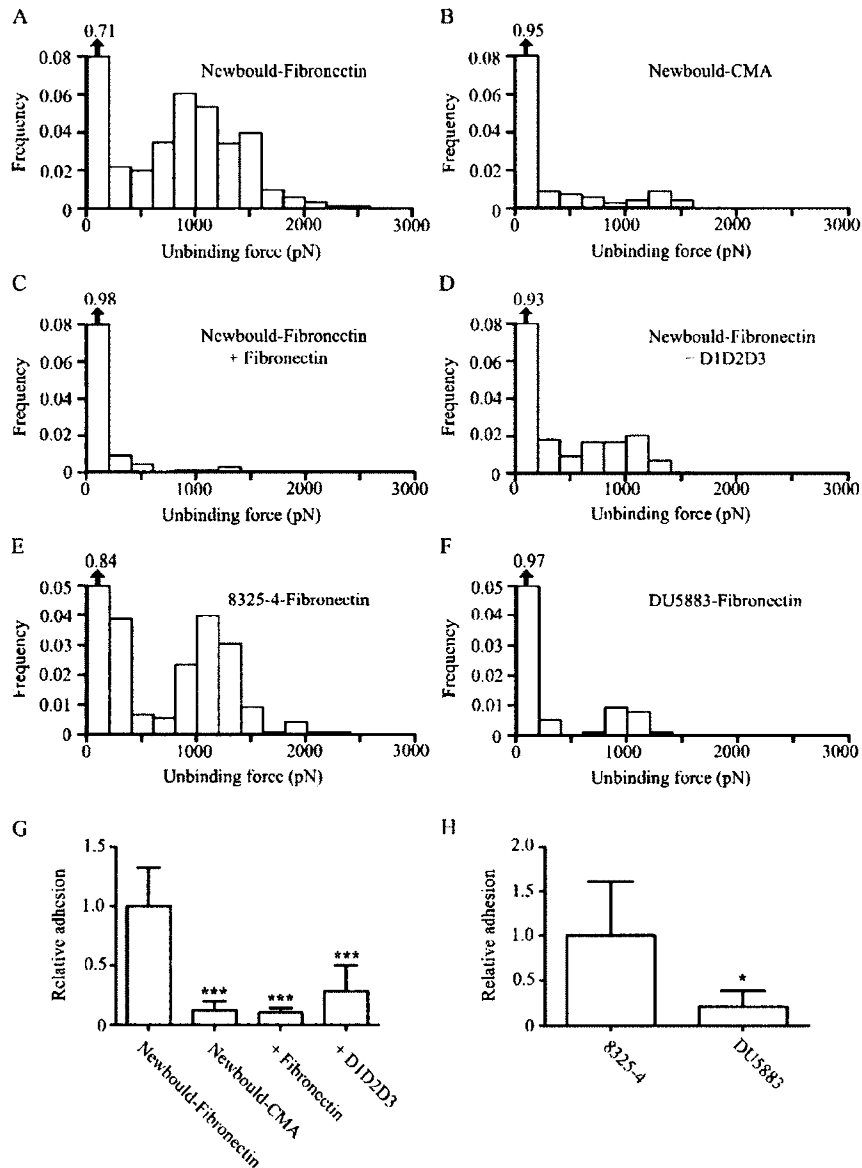

FIGURE 2. The dissociation of fibronectin-*S. aureus* FnBPs complexes requires strong mechanical forces. Histograms present unbinding forces of the interaction between fibronectin and Newbould (n=1559 retraction curves from 8 independent experiments) (A), between carboxymethylamylose (CMA) and Newbould (n=632 from 4 independent experiments) (B)

and between fibronectin and Newbould in the presence of free fibronectin (n=603 from 3 independent experiments) (C) and free D1D2D3 (n=817 from 4 independent experiments) (D). Histograms present unbinding forces of the interaction between fibronectin and the *fnb*$^+$ strain 8325-4 (n=849 from 4 independent experiments) (E) and the *fnbAB* mutant (DU5883) (n=731 from 4 independent experiments) (F). Numbers beside the arrows correspond to the total frequency of rupture events between 0 and 200 pN. Relative adhesions of the AFM probe to Newbould for different experiments are compared (G). Relative adhesions of fibronectin to 8325-4 and DU5883 are also shown (H). The results have been normalized with the adhesion obtained for Newbould or 8325-4. Data were obtained from bacteria in early exponential growth. Standard deviations and significant differences compared to relative adhesions of Newbould or 8325-4 to fibronectin are indicated (*, $P < 0.05$; ***, $P < 0.001$).

Mechanical characterization of the fibronectin-FnBPs interaction by dynamic force spectroscopy. AFM-based force spectroscopy applies an external force on the molecular interactions studied. This external force decreases the activation energy needed to reach the transition state of dissociation and exponentially increases the rate of dissociation of an interaction (Bell, 1978). The resistance and the compliance of a particular molecular interaction to a mechanical load can thus be measured by varying the applied force. Since there is a correlation between the loading rate of an applied force and the mean unbinding force of a particular molecular interaction, it is possible to study the mechanical response of an interaction to various external forces by doing experiments at different loading rates (see Experimental Procedures) (Evans and Ritchie, 1997; Mitchell *et al.*, 2007). Fig. 3 presents the dynamic force spectrum or the mean unbinding force as a function of the logarithm of the mean loading rate of the fibronectin-FnBPs interaction obtained with the Newbould strain in early exponential growth phase. Variation of loading rates from 7000 ± 3000 pN/s to 300000 ± 100000 pN/s resulted in an increase of the mean unbinding forces from 1080 ± 50 pN to 1300 ± 100 pN. These data fit (R^2= 0.71) with the model established for a molecular interaction with one linear regime of mean rupture forces as a function of the logarithm of the mean

loading rates. However, it is possible that other regimes may appear at loading rates not tested here. While it is possible to isolate an energetic barrier width χ_B of 0.6 ± 0.1 Å from the equation describing the fibronectin-FnBPs dynamic force spectrum, it is impossible to obtain a precise k_{off} because of the related error. However, this k_{off} is obviously below 2 x 10 $^{-4}$ s^{-1}. The value χ_B describes the deformation that an energetic barrier can sustain (Liphardt *et al.*, 2001) whereas the k_{off} is inversely linked to its lifetime.

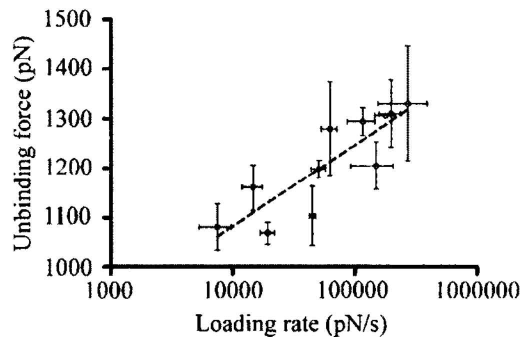

FIGURE 3. Dynamic force spectrum of the fibronectin-FnBPs interaction. Mean values of the unbinding forces and loading rates are plotted with their standard errors of the mean. The correlation between mean unbinding forces and the logarithm of the mean loading rates is described by the equation $F = (70 \pm 10)\ ln\ L_r - (400 \pm 200)$ and a correlation factor of 0.74. Data were obtained from the Newbould strain in early exponential growth.

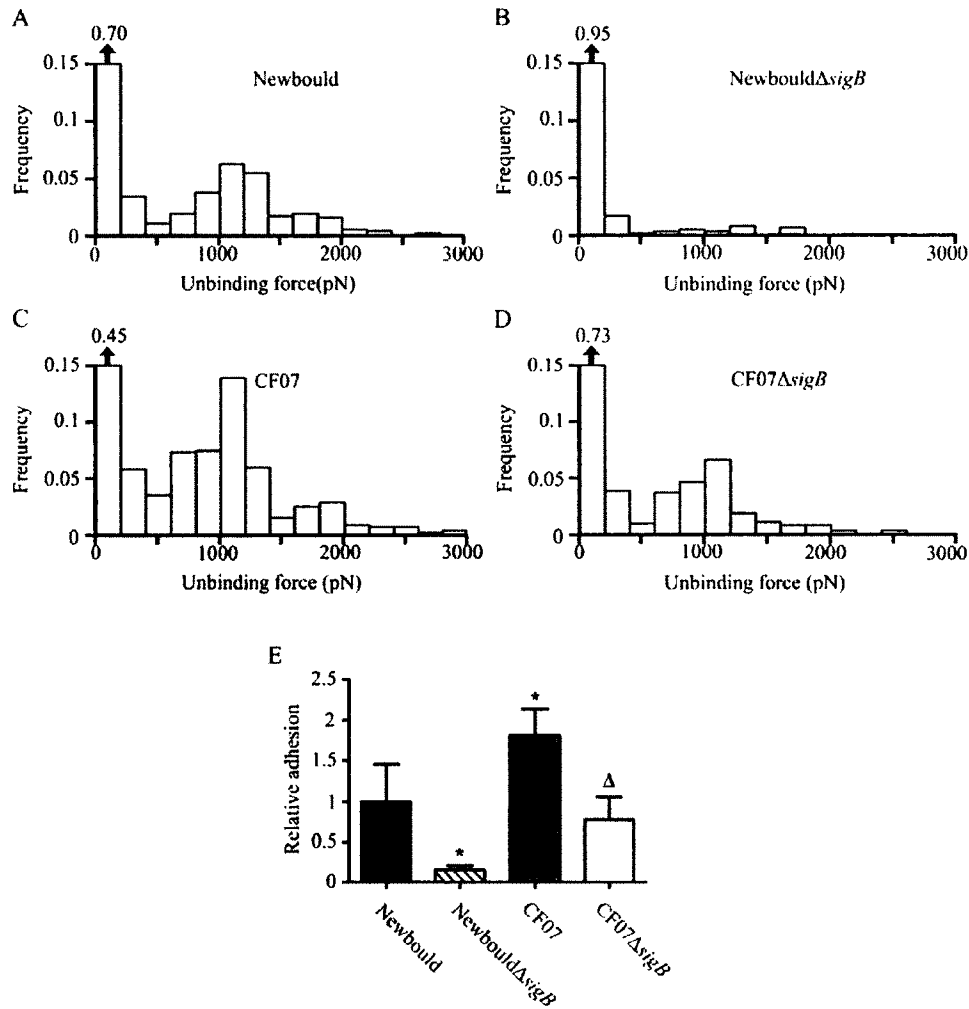

FIGURE 4. *S. aureus* SigB activity promotes a strong fibronectin-bacteria interaction. Histograms present unbinding forces of the interaction between fibronectin and Newbould (n=932 from 6 independent experiments) (A), NewbouldΔ*sigB* (n=663 from 3 independent experiments) (B), CF07 (n= 607 from 3 independent experiments) (C) and CF07Δ*sigB* (n=653 from 3 independent experiments) (D) in early exponential growth. Numbers beside arrows correspond to the total frequency of rupture events between 0 and 200 pN. Relative adhesions of Newbould, NewbouldΔ*sigB*, CF07 and CF07Δ*sigB* to fibronectin are shown (E). Results

have been normalized with the adhesion obtained for Newbould. Standard deviations and significant differences compared to the relative adhesion of Newbould (*, $P < 0.05$) or CF07 (Δ, $P < 0.05$) to fibronectin are indicated.

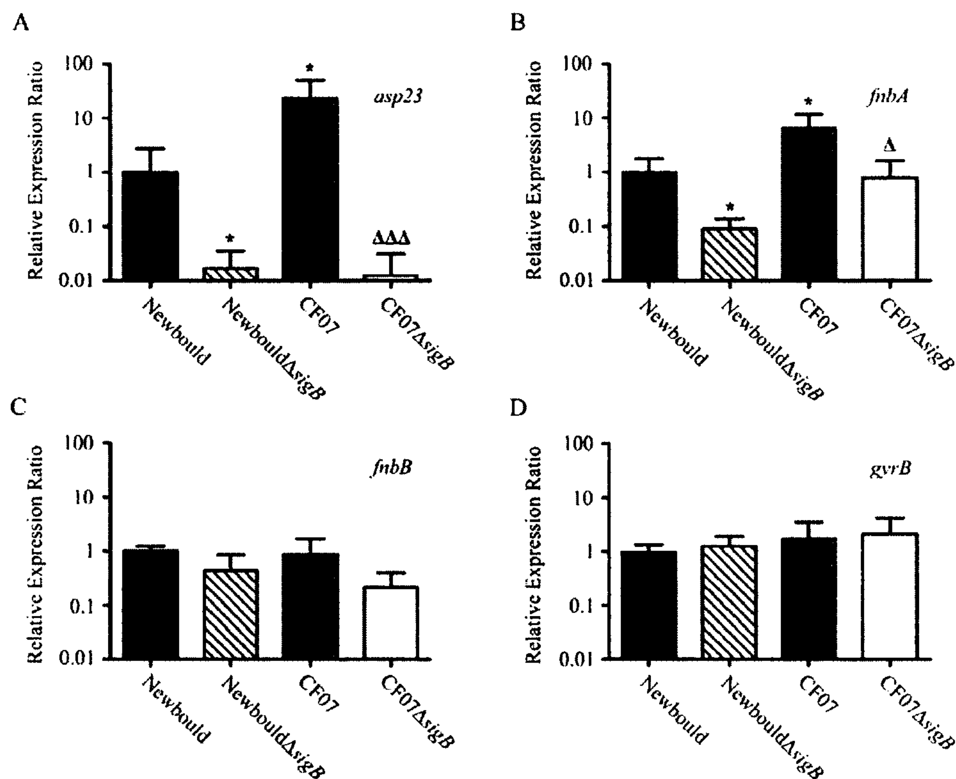

FIGURE 5. The expression of *fnbA* is influenced by SigB activity in early exponential growth. Expression ratios (test strain/Newbould) for the genes *asp23* (A), *fnbA* (B), *fnbB* (C) and *gyrB* (D) were evaluated by real-time PCR for strains Newbould, Newbould*ΔsigB*, CF07 and CF07*ΔsigB* in the early exponential growth phase. Data are presented as means and standard deviations from three independent experiments. Significant differences compared to Newbould (*, $P < 0.05$) and CF07 (Δ, $P < 0.05$; ΔΔΔ, $P < 0.001$) are indicated.

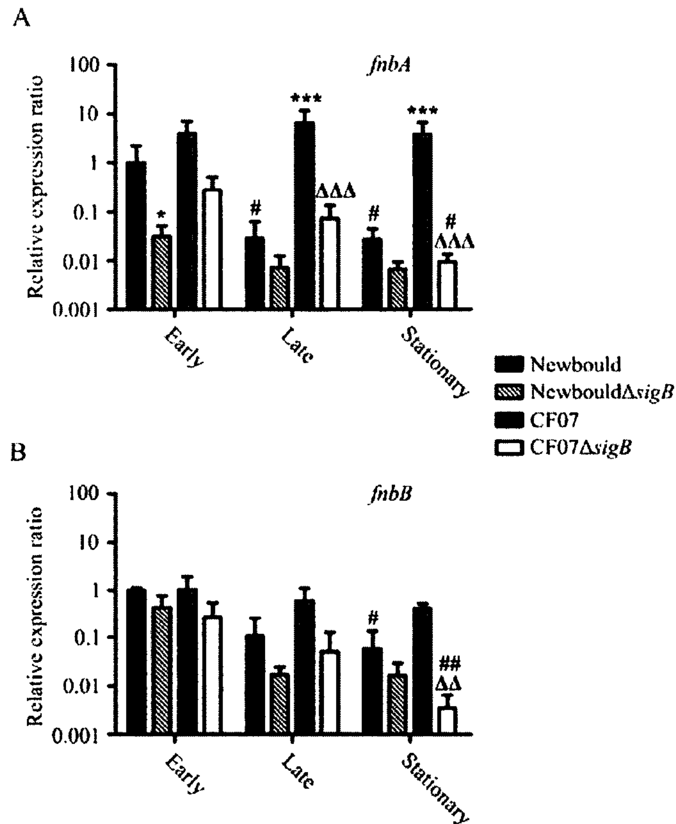

FIGURE 6. The expression of *fnbA* is locked throughout the growth of CF07. Expression ratios (test strain/Newbould in early exponential) for the genes *fnbA* (A) and *fnbB* (B) were evaluated by real-time PCR over the early exponential, late exponential and stationary phases of growth for strain Newbould, NewbouldΔ*sigB*, CF07 and CF07Δ*sigB*. Data are presented as means and standard deviations from three independent experiments. Significant differences within growth phases in comparison to Newbould (*,$P < 0.05$; ***, $P < 0.001$) or CF07 (ΔΔ, $P < 0.01$; ΔΔΔ, $P < 0.001$) and in comparison to the early exponential growth phase of each strains (#, $P < 0.05$; ##, $P < 0.01$) are shown.

***S. aureus* SigB activity promotes a strong fibronectin-bacterium interaction.** The importance of SigB in the ability of *S. aureus* to bind fibronectin was also evaluated using AFM-based force spectroscopy during the early exponential growth phase. Newbould and CF07 strains were, respectively, used as a prototype strain and as a clinical SCV with high SigB activity (Moisan *et al.*, 2006). Furthermore, *sigB* knock-outs (Δ*sigB*) were generated in these backgrounds. We already validated the choice of Newbould as a comparator strain (Moisan *et al.*, 2006). Newbould indeed behaves like other prototype strains according to growth rate and expression of virulence factors. Fig. 4A represents a second set of experiments which characterized the interaction between fibronectin and the Newbould strain. A force signature with a mean value of 1100 ± 500 pN was observed, a result highly similar to that presented in Fig. 2A. Fig. 4B-D present unbinding forces histograms of fibronectin interacting with NewbouldΔ*sigB*, CF07 and CF07Δ*sigB*, respectively. CF07 and CF07Δ*sigB* also showed force groups with mean values of 1100 ± 500 pN and 1000 ± 500 pN, respectively. CF07 interacted with the fibronectin at a higher level than Newbould and the interaction frequency of both *sigB* mutants were lower than their parental strains. The amplitude of the fibronectin-binding differences between these strains was best visualized by plotting their relative adhesion (Fig. 4E). Interestingly, the inactivation of *sigB* in Newbould almost completely eliminated fibronectin interactions, whereas CF07Δ*sigB* still significantly binds fibronectin suggesting the involvement of additional regulators for the expression of FnBPs. Note that our results do not allow us to identify the binding frequency of individual bacteria but instead relate to the overall effect of SigB activity on the occurrence of the fibronectin-FnBPs interaction in the cell population of a given strain.

In order to confirm our observations and to further define the relationship between the frequency of fibronectin-binding, transcription of *fnb* genes and SigB activity, we conducted real-time PCR measurements to evaluate the expression of *asp23*, *fnbA* and *fnbB* in the early exponential growth phase. The regulation of SigB activity involves multiple protein-protein interactions which respond to a variety of stimuli (Price, 2002). Thus, the expression level of

sigB is not necessarily representative of bacterial SigB activity. We therefore chose to examine the expression level of the *asp23* gene, a well-known indicator of SigB activity (Bischoff *et al.*, 2004; Moisan *et al.*, 2006). Fig. 5A presents the relative expression ratio of *asp23* which was indeed significantly reduced in both *sigB*-minus strains. Moreover, we were not able to directly detect *sigB* expression in the mutants (data not shown). We also confirmed the previously shown elevated expression of *asp23* (SigB activity) in CF07 compared to that found in the Newbould strain (Moisan *et al.*, 2006). The *fnbA* gene is also under the influence of SigB (Bischoff *et al.*, 2004; Entenza *et al.*, 2005; Senn *et al.*, 2005) and, as expected, expression patterns for *fnbA* were similar to those observed for *asp23* (Fig. 5B). However, the expression of *fnbB* was not significantly different between strains (Fig. 5C), which suggests that SigB activity has a greater impact on *fnbA* than on *fnbB* expression during the early exponential growth phase. The expression of the housekeeping gene *gyrB* (Fig. 5D) has also been measured for comparison. Interestingly, the expression ratios of *asp23* and *fnbA* correlate best with the AFM-based force spectroscopy results.

The expression of the *fnbA* gene and the fibronectin-binding ability of the clinical SCV strain are sustained throughout growth. The expression patterns of *fnbA* (Fig. 6A) and *fnbB* (Fig. 6B) at different growth phases were obtained by real-time PCR for the prototypic strain Newbould, NewbouldΔ*sigB*, the clinical SCV CF07 and CF07Δ*sigB*. FnBPs are usually down-regulated after the early exponential growth phase in prototypic strains (Saravia-Otten *et al.*, 1997) as indeed observed for Newbould (Fig.6). On the other hand, *fnbA* was stably expressed at relatively high levels in CF07 (Fig. 6A). The inactivation of *sigB* in the CF07 background resulted in a down-regulation of the *fnbA* expression at all time points studied (statistically significant for the late exponential and stationary growth phases) which then followed a more prototypic pattern of expression. The expression of *fnbB* was not at higher levels in CF07 compared to Newbould for all time points studied and was only significantly down-regulated in the stationary phase of growth in CF07Δ*sigB* (in comparison to CF07). This

suggests that the sustained activity of *sigB* increases and locks the expression level of *fnbA* throughout the growth of CF07.

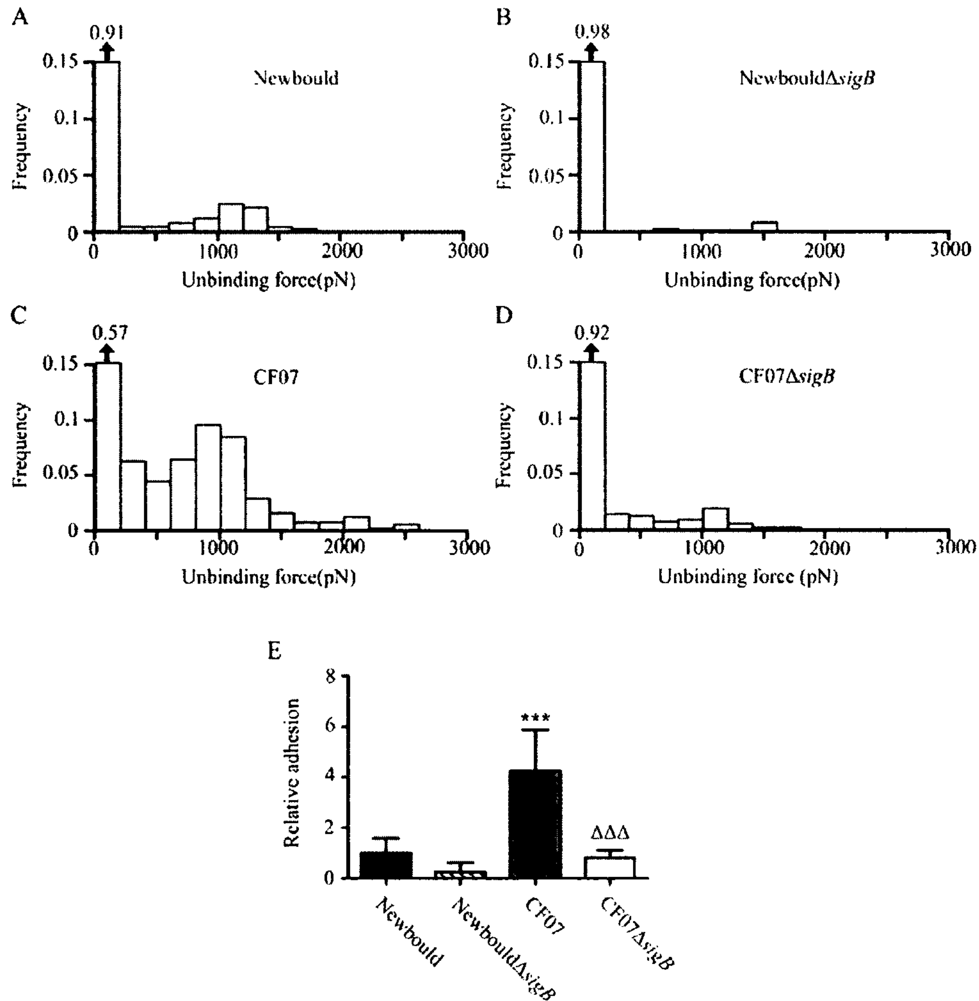

FIGURE 7. The fibronectin-binding ability of CF07 is sustained up to the stationary growth phase. Histograms present unbinding forces of the interaction between fibronectin and Newbould (n=1026 from 6 independent experiments) (A), NewbouldΔ*sigB* (n=761 from 4 independent experiments) (B), CF07 (n= 606 from 3 independent experiments) (C) and

CF07Δ*sigB* (n=606 from 3 independent experiments) (D) in the stationary growth phase. Numbers beside arrows correspond to the total frequency of rupture events between 0 and 200 pN. Relative adhesions of Newbould, NewbouldΔ*sigB*, CF07 and CF07Δ*sigB* to fibronectin in the stationary growth phase are shown (E). Results have been normalized with the adhesion obtained for Newbould. Standard deviations and significant differences compared to the relative adhesion of Newbould (***, $P < 0.05$) or CF07 (ΔΔΔ, $P < 0.05$) to fibronectin are indicated.

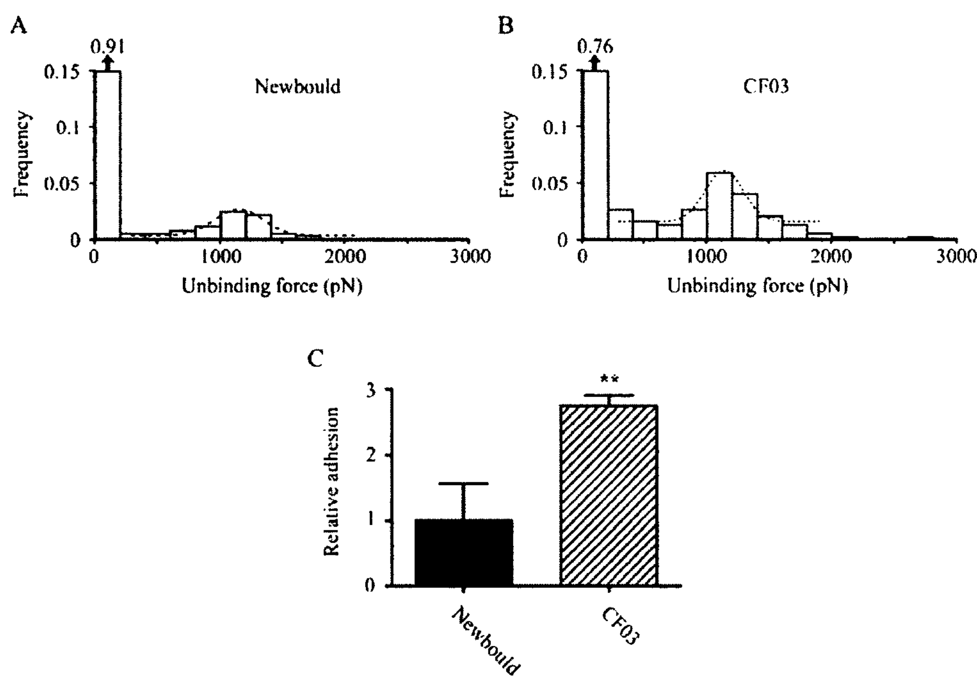

FIGURE S4. Comparison of the fibronectin-binding ability of Newbould and CF03 in the stationary growth phase. Histograms present unbinding forces of the interaction between fibronectin and Newbould (n=932 from 6 independent experiments) (A) and CF03 (n=653 from 3 independent experiments) (B) in the stationary growth phase. Numbers beside the arrows correspond to the total frequency of rupture events between 0 and 200 pN. Relative

adhesions of Newbould and CF03 to fibronectin in the stationary growth phase are shown (C). Results have been normalized with the adhesion obtained for Newbould. Standard deviations and significant differences compared to the relative adhesion of Newbould to fibronectin are indicated (**, $P < 0.01$ from an unpaired *t*-test).

Fig. 7 presents histograms of unbinding forces as well as relative adhesions recorded on Newbould, NewbouldΔ*sigB*, CF07 and CF07Δ*sigB* strains in the stationary phase of growth using fibronectin coated AFM probes. Under this condition, Newbould (Fig. 7A) bound fibronectin at a much lower frequency than that observed when this strain was used in the early exponential phase of growth (Fig. 2A and 4A) and was not binding fibronectin significantly more than NewbouldΔ*sigB* (Fig. 7B). A force population defined by a mean of 1000 ± 300 pN can barely be detected with Newbould. On the other hand, a force group of 900 ± 500 pN was clearly observed in the CF07 distribution (Fig. 7C, Table 1) at a frequency close to that observed in early exponential growth (Fig. 3C). The observed binding to fibronectin was abolished in CF07Δ*sigB* (Fig. 7D). Significant differences between the relative adhesions of the strains are reported in Fig. 7E. This suggests that the sustained expression of *fnbA* in CF07 allows strong fibronectin-binding from the early exponential to the stationary phase of growth. The fibronectin-binding frequency of another independent cystic fibrosis SCV (isolate CF03) is also higher than that of Newbould in the stationary phase of growth (Fig. S4). This suggests that the sustained fibronectin-binding ability of CF07 may represent a common feature of SCV isolates from cystic fibrosis patients.

DISCUSSION

This study defines the potential mechanical contribution of the fibronectin-FnBPs interaction in *S. aureus* adhesion to host tissues. We found that the mean rupture force of the fibronectin-

FnBPs interaction is around 1000 pN at a pulling rate of 2 µm/s. Table 1 shows adhesion frequencies and mean unbinding forces between fibronectin and different *S. aureus* strains for the growth phases studied. The mean forces of dissociation were not significantly variable between strains. However, the frequency of fibronectin-FnBPs unbinding events seemed to be strain dependant and proportional to the level of expression of *fnb* genes. This confirms that variations in FnBPs expression alter the global fibronectin-binding frequency of a particular bacterial population whereas it has no impact on the fundamental force related to the dissociation of fibronectin-FnBP complexes. Besides, the variance of the measured unbinding forces can be explained by the simultaneous presence of both FnBPs (FnBPA and FnBPB) on the bacterial surface and of multiple fibronectin-binding sites of different affinities on each of these FnBPs. It has recently been reported that FnBPs have 10 to 11 fibronectin-binding sites and that 6 of them have a dissociation constant in the nanomolar range whereas it is in the micromolar range for the others (Meenan *et al.*, 2007). Furthermore, the dissociation of a molecular complex under equilibrium condition is a stochastic process which is influenced by the thermal energy of the system and is modified by an applied force. In this context, before one of the molecular partners is pulled off, the complex can present itself in several different transitory states of association which may be of different strengths. Here, we can anticipate that the highly complex interaction between FnBPs and fibronectin will present a wide number of transitory states, which may result in the broadening of the unbinding force distribution. Moreover, during the course of our experiments, the complex was potentially in variable orientations relative to the pulling direction of the AFM probe. This may also lead to the broadening of the unbinding forces measured. The dependence of unfolding forces of β-sheet proteins on the pulling direction has indeed been previously noted in several studies (Evans, 2001; Brockwell *et al.*, 2003) and it has been shown that the mechanical unbinding of the amyloid β-peptide from amyloid fibrils is anisotropic (Raman *et al.*, 2007).

TABLE 1. Adhesion frequencies and mean unbinding forces between fibronectin and different *S. aureus* strains at the indicated growth phases.

Strain	Early exponential Adhesion Frequency[a]	Early exponential Unbinding force (pN)[b]	Stationary Adhesion Frequency[a]	Stationary Unbinding force (pN)[b]
Newbould	0.29	1100 ± 500	0.09	1000 ± 300
Newbould∆*sigB*	0.05	800 ± 500	0.02	1100 ± 300
8325-4	0.16	900 ± 500	N.D.	N.D.
DU5883	0.03	800 ± 300	N.D.	N.D.
CF07	0.55	1100 ± 500	0.43	900 ± 500
CF07∆*sigB*	0.27	1000 ± 500	0.08	900 ± 500
CF03[c]	N.D.	N.D.	0.24	1100 ± 500

[a]Overall adhesion frequencies expressed as the probability to observe specific unbinding events between 200 and 3000 pN. [b]Mean unbinding forces ± standard deviations were calculated by considering the specific unbinding events between 200 and 3000 pN. [c]See Fig. S4. N.D., not determined.

The rupture force of the fibronectin-FnBPs interaction is high compared to that required to break most of the molecular recognition pairs measured by AFM-based force spectroscopy (for review see Zlatanova *et al.*, 2000; Lee *et al.*, 2007). The strength of several ligand-receptor interactions is effectively found to be around 100 pN (Busscher *et al.*, 2008). However, many of the ligand-receptor interactions already studied by force spectroscopy can either be considered as simple interactions involving small molecules and thus a limited number of intermolecular interactions (*i.e.*, the streptavidin-biotin interaction) or interactions requiring reversible binding to fulfill biological functions such as the interaction between

actin-binding proteins and actin filaments (Ferrer *et al.*, 2008) or fibronectin and integrins (Sun *et al.*, 2005). On the other hand, protein-protein interactions can be more complex, involving several type of interactions, such as hydrogen bounding, saline bridges and hydrophobic residue partitioning all occurring in a larger interface of interaction. There are indeed examples of protein-protein interactions structurally similar to the fibronectin-FnBPs interaction (*i.e.*, involving β-sheets addition as part of the mechanism of interaction) that have been shown to have high unbinding forces (Yip *et al.*, 1998, McAllister *et al.*, 2005; Raman *et al.*, 2007).

McAllister *et al.* (2005) used the AFM to study homo-interactions of three unrelated and structurally distinct proteins, amyloid β-peptide, α-synuclein, and lysozyme. At low pH, they obtained unbinding forces as high as 300 pN, 850 pN and 1200 pN for the amyloid β-peptide, α-synuclein and the lysozyme, respectively (at a pulling rate of 0.25 μm/s). Interestingly, it was demonstrated that the increase in unbinding forces was due to a conformational transition in the amyloid β-peptide and α-synuclein proteins leading to the formation of a structure with an elevated content of β-sheet conformations. Interestingly, the formation of extensive β-sheet structures by fibrillization of polypeptides is a common feature of amyloids, which are remarkably stable against denaturation and are essentially irreversible under physiological conditions (Serpell, 2000; Hamada and Dobson, 2002). In the case of the interaction between amyloid β-peptides and amyloid fibrils, the mechanical unbinding characteristics are determined by a combination of intrafibril interactions including Lys-Asp electrostatic contacts, hydrophobic interactions and backbone H-bonds. According to steered-molecular dynamics simulations, these result in a stability greater than most protein domains (Raman *et al.*, 2007). Molecular dynamics simulations have also shown that the insulin-insulin complex is held together by numerous hydrophobic interactions and H-bonds (Yip *et al.*, 1998). Moreover, some residues involved in dimerization adopt a β-sheet structure and the dimer-forming interface is formed by an antiparallel contact between these β-sheets (Blundell *et al.*,

1972). Yip *et al.* (1998) found that a force of approximately 1300 pN is required to break this interaction.

The unusual strength of the interaction taking place between fibronectin and FnBPs finds its rationale in many studies revealing its complexity. The existence of several repeats of FnBP-binding domains along the fibronectin has been reported by Hayashi and Yamada (1981). In addition, Bozzini *et al.* (1992) demonstrated that both the N-terminal and the heparin-binding domains of fibronectin bind the staphylococcal receptors. These authors speculated that multiple fibronectin-binding sites cooperated in a synergistic mechanism by which the binding of one domain enhances the interaction of the other domains. It has also been proposed that the bacterial FnBPs exploit the modular structure of fibronectin by forming extended tandem β-zippers during the interaction (Shwarz-Linek *et al.*, 2003). Extension of β-sheets (β-zipper) during intermolecular interactions has been observed before (for review see Remaut and Waksman, 2006), but the fibronectin-FnBPs interaction is probably the only one known to involve the formation of β-strands on adjacent domains (extended tandem β-zippers). Sequence analyses of regions of FnBPs from *Streptococcus pyogenes* and *S. aureus* indeed revealed a repeating pattern of F1-binding motifs that match the pattern of F1 modules in the $^{1\text{-}5}$F1 fibronectin region (Shwarz-Linek *et al.*, 2003). This was confirmed by NMR spectroscopic analysis of the complex formed by fibronectin ^{1}F-^{2}F peptide and the B3 domain of *Steptococcus dysgalactiae,* which demonstrated Hα-Hα intermolecular distances typical of β-sheet formation. In *S. aureus*, up to four antiparallel β-sheets can be formed with the $^{1\text{-}5}$F1 fibronectin region at high affinity fibronectin binding sites of FnBPs. Furthermore, the binding of FnBPs to fibronectin is predicted to also involve hydrophobic and electrostatic interactions (Ingham *et al.*, 2004). The nature of the interaction between FnBPs and fibronectin may thus explain its high resistance to mechanical load.

Dynamic force spectroscopy experiments were also conducted in order to further characterize the mechanical properties of this interaction. We have found that an energetic barrier width of 0.6 ± 0.1 Å and an off-rate below 2 x $10^{-4}s^{-1}$ describe the compliance and the resistance of this interaction under load, respectively. Whereas the energetic barrier width of this interaction is of a common magnitude, its resistance is high when compared to other molecular recognition pairs already studied by dynamic force spectroscopy (for review see Lee *et al.*, 2007). Dissociation rates for receptor-ligand interaction are typically in the range of 0.01-10 s^{-1} (Thoumine *et al.*, 2000). Bustanji *et al.* (2003) found an off-rate of 4.8 s^{-1} for the fibronectin-*Staphylococcus epidermidis* interaction. Despite the high structural homology between *S. aureus* and *S. epidermidis* FnBPs, previous results support the idea that *S. aureus* is much more adherent to immobilized fibronectin than *S. epidermidis* (Vaudaux *et al.*,1993) and it was suggested that the fibronectin-*S. aureus* interaction might be irreversible under physiological conditions (Proctor *et al.*, 1982). Indeed, Holmes *et al.* (1997) were barely able to detect binding of *S. epidermidis* to fibronectin by using surface plasmon resonance while the dissociation of *S. aureus* following fibronectin-binding was not possible to achieve. The limit of k_{off} measurement of their system was approximately 10^{-5} s^{-1}. These authors have also shown that *S. aureus* preferentially binds to the N-terminal region of fibronectin whereas *S. epidermidis* binds to the C-terminal domain. Joh *et al.* (1994) found a k_{off} of 7.8 ± 0.8 x 10^{-4} s^{-1} between the D1D2D3 recombinant fibronectin-binding fragment of *S. aureus* and fibronectin, which also corroborates our results.

Yongsunthon *et al.* (2007) indeed reported fibronectin-*S. aureus* unbinding events obtained using AFM-based force spectroscopy in the same force range as us. Furthermore, *in vitro* and *in vivo* studies have demonstrated that the presence of FnBPs on the surface of *S. aureus* increased the ability of this bacterium to colonize tissues under physiological shear stresses (Reddy and Ross, 2001; Brouillette *et al.*, 2003). These findings suggest that the fibronectin-FnBPs interaction efficiently resists physiological mechanical stresses and allows the bacterium to remain attached to host tissues. The large magnitude of the force measured here

also supports the hypothesis that *S. aureus* is so tightly attached to fibronectin that it actually needs to use exoproteases on its own adhesins to disseminate from colonization sites (Karlsson *et al.*, 2001).

SigB activity has been associated with an increase in *S. aureus* adherence to host cells and extracellular matrix components (Entenza *et al.*, 2005). Since the expression of *fnbA* was shown to be positively influenced by SigB (Bischoff *et al.*, 2004; Entenza *et al.*, 2005; Senn *et al.*, 2005), we propose that the increased ability of CF strains to adhere to cells and matrix components is, at least in part, due to an increase in FnBPA expression. Interestingly, Inose *et al.* (2006) found natural variant strains having higher concentrations of SigB in methicillin-resistant *S. aureus* (MRSA) and we have shown that *S. aureus* SCVs isolated from cystic fibrosis patients have a strong SigB activity (Moisan *et al.*, 2006). SigB could then be related to particular chronic or difficult-to-treat infections. The results presented here support the hypothesis that SigB activity locks CF-isolated SCVs into a colonization state, which is likely to contribute to the establishment of chronic infections.

Our results also indicate that the deletion of *sigB* may have different effects depending on the genetic background of the strain used. Indeed, the inactivation of *sigB* in Newbould almost completely abolished the fibronectin-binding ability of this strain in the early exponential growth phase, whereas a significant fibronectin-binding ability still remained when this inactivation was realized in CF07. This may be easily explained if other regulators also influence the expression of FnBPs, which is indeed the case in *S. aureus* where the Agr system is known to down-regulate the expression of FnBPs (Saravia-Otten *et al.*, 1997). This reliance on both control mechanisms may explain the more drastic effect of the *sigB* deletion in the prototypic strain Newbould that was shown to normally activate the Agr system in comparison to the clinical SCV strain CF07, which failed to show significant expression of the *agr* locus (Moisan *et al.*, 2006). Interestingly, the expression of the V8 protease is under the control of

Agr (Arvidson and Tegmark, 2001) and it was reported that this protease is the most important in the release of cell-bound FnBPs (Karlsson *et al.*, 2001). It is thus possible that a synergism between the sustained *fnbA* expression and the down-regulation of the V8 protease combines to increase the fibronectin-binding ability of the cystic fibrosis isolated SCVs.

It is tempting to speculate that the physiological benefit to have two *fnb* genes is to allow a more subtle and complex regulation of their expression. Li *et al.* (2005) have provided evidences that the induction of FnBPs by SigB-mediated and ciprofloxacin-triggered responses involve separate regulatory networks and that the RecA-LexA pathway induces fibronectin-binding by the up-regulation of *fnbB*. Furthermore, it has been shown that SarA is up-regulating the transcription of *fnbA* but not *fnbB* (Wolz *et al.*, 2000; Xiong *et al.*, 2004). The expression of *fnb* genes can thus be induced individually following different stimuli. However, the Agr, SarA and SigB regulatory networks are not totally independent and it has been demonstrated that SigB increases *sarA* expression while simultaneously reducing RNAIII expression (Bischoff *et al.*, 2001). It is then possible that the effect of SigB activity on the expression of *fnb* genes observed here is not direct and implies other regulatory networks.

Several lines of research suggest that SCVs from other microorganisms are associated with certain types of infection. Interestingly, *S. aureus* and *Pseudomonas aeruginosa* SCVs have been linked to chronic infections in CF patients (Zierdt & Schmidt, 1964; Kahl *et al.*, 1998; Haussler *et al.*, 1999; Sadowska *et al.*, 2002). SCVs from *Stenotrophomonas maltophilia* have also been isolated from the sputum of five CF patients presenting persistent infections (Anderson *et al.*, 2007). Haussler *et al.* (2003) have isolated *P. aeruginosa* SCVs in CF-infected airways, which were highly adherent and similarly, SCVs from *Streptococcus pneumoniae* biofilms were found to have a marked adherence (Allegrucci & Sauer, 2007). These observations suggest that SCVs of many pathogens may have a selective advantage in CF airways and are involved in the establishment of chronic infections. Multiple mechanisms

are likely to play a role in the overall pathogenesis of SCVs but an increased and sustainable adherence may represent one common feature of persistent bacteria able to successfully challenge the compromised mucociliary clearance of CF airways.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

S. aureus Newbould (ATCC 29740) was used as a representative control (prototype strain) for the wild-type phenotype (Moisan *et al.*, 2006). An isogenic mutant of *S. aureus* Newbould, NewbouldΔ*sigB*, in which *sigB* had been disrupted by the *emrA* cassette (Moisan *et al.*, 2006), was used to evaluate the importance of *sigB* in a prototypic background. Isolates CF03 and CF07, both having elevated intrinsic SigB activity, are previously characterized independent menadione-auxotroph SCVs recovered from CF patients (Moisan *et al.*, 2006). The strains 8325-4 and DU5883 (*fnbAB* mutant) were kindly provided by Dr. T. J. Foster (Moyne Institute of Preventive Medicine, Dublin, Ireland). DU5883 is an isogenic strain of 8325-4 that has been mutated by the insertion of a tetracycline resistance cassette in *fnbA* and an erythromycin resistance cassette in *fnbB* (Greene *et al.*, 1995). For all experiments presented in this paper, cultures grown overnight were used to inoculate brain heart infusion broth at A_{595nm} of 0.1. The bacteria were then grown aerobically at 35°C until they reached a specific growth phase. The A_{595nm} values for the early and the late exponential growth phases were, respectively, 0.4 and 3.0-3.5 for the prototype strains and, 0.250 and 0.9-1.0 for the SCV strains (Moisan *et al.*, 2006). The stationary phase samples were taken after 12 h of growth.

Construction of *S. aureus* CF07Δ*sigB*

An isogenic mutant of the CF07 strain was constructed, in which the *sigB* gene was disrupted by the insertion of the *emrA* cassette by homologous recombination. The temperature-sensitive

pBT2-*sigB*::*emrA* plasmid was used combined to a strategy previously described (Bruckner, 1997; Moisan *et al.*, 2006). Briefly, pBT2-*sigB*::*emrA* was transferred for propagation into RN4220 (*res*$^-$). After bacterial lysis with lysostaphin (200 µg/ml for 1 h at room temperature), plasmid DNA was then isolated using the QIAfilter Plasmid Midi kit (QIAGEN, ON, Canada) and used to transform CF07 by electroporation. Bacteria were firstly grown overnight at 30°C with 10 µg/ml of erythromycin and 1 µg/ml of menadione (Sigma-Aldrich, ON, Canada). Bacteria were then diluted 1:1000 and grown overnight at 40°C with 2.5 µg/ml of erythromycin and 1 µg/ml of menadione. This step was repeated once. Finally, bacteria were diluted 1:1000 and grown overnight at 40°C with1 µg/ml of menadione. Homologous recombinants with the inactivated *sigB* gene were selected as resistant to erythromycin and sensitive to chloramphenicol. The knockout of *sigB* in the strain CF07Δ*sigB* was confirmed by PCR.

***S. aureus* immobilization on NHS-activated glass slides**

Bacteria were covalently bound to glass slides using carbodiimide chemistry without being in direct contact with chemical agents. Clean glass slides were incubated for 5 h at 80°C and 23 mm Hg vacuum in the presence of *N*'-[3-(trimethoxysilyl)-propyl]-diethylenetriamine (Sigma-Aldrich) vapor, rinsed with distilled water and cured for 1 h at 80°C and 23 mm Hg vacuum. A drop of 500 µl of a 40 mM citrate (Laboratoire Mat, QC, Canada) solution, pH 5.3, activated with 100 mg/ml of *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) (Sigma-Aldrich) and 40 mg/ml of *N*-hydroxysuccinimide (NHS) (Sigma-Aldrich) was added to the amino-functionalized slides and incubated for 15 min at room temperature. Glass slides were washed rigorously with phosphate buffered saline (PBS) and a 500 µl drop of concentrated bacterial culture at approximately 10^{10} CFU/ml was incubated on the activated glass slides for 40 min at room temperature. Bacteria were then washed several times with PBS and incubated for 15 min in tris-buffered saline (138 mM NaCl, 2.7 mM KCl and 25 mM Tris-Base, pH 7.3) in order to quench the remaining reactive groups on the glass slides. Bacteria coated slides were

kept in PBS and at 4°C for a maximum of 12 h until use. This did not significantly alter the viability of the bacteria (Fig. S1) and the unbinding forces recorded (Fig. S2).

Fluorescence microscopy

The immobilization efficiency and the viability of *S. aureus* Newbould covalently attached to glass slides were evaluated using LIVE/DEAD® BacLight™ Bacterial Viability Kits (Molecular Probes, OR, USA) as described by the manufacturer.

Fibronectin functionalization of AFM tips

Human plasma fibronectin (Calbiochem, ON, Canada) was covalently attached to silicon nitride tips (Veeco, CA, USA) using carboxymethylamylose (CMA) spacers (Sigma-Aldrich) and the carbodiimide chemistry as previously described (Mitchell *et al.*, 2007). The CMA was used as a molecular spacer because non-specific interactions between the bacteria and the CMA-modified probe without fibronectin were rare and did not have the same properties as those of the studied interaction. The CMA is also likely to allow the high mobility of the anchored molecules and to be in a highly hydrated state. This may mimic the physiological environment of soluble proteins. Briefly, the tips were dipped in 12.5% hydrofluoric acid (Fischer scientific, ON, Canada) for 30s, rinsed with water and incubated for 10 min in concentrated nitric acid (Fischer scientific). The tips were then incubated over an *N'*-[3-(trimethoxysilyl)-propyl]-diethylenetriamine bath for 4 h at 80°C and 23 mm Hg vacuum. After the amino-silanization, cantilevers were rinsed in distillated water and cured for 1 h at 80°C and 23 mm Hg vacuum. A 10 mg/ml CMA solution in PBS was activated with 50 mg/ml of EDC and 20 mg/ml of NHS. The amino-functionalized tips were incubated in this solution for 15 min, thoroughly rinsed in PBS and incubated for 1 h with 1 mg/ml of fibronectin at room temperature. Excess reactive groups on the CMA functionalized tips were quenched with 1M ethanolamine (Sigma-Aldrich), pH 8.5, for 10 min at room temperature. Fibronectin functionalized tips were kept at 4°C for a maximum of 12 h until use.

AFM-based force spectroscopy

All AFM experiments were performed with a soft silicon-nitride cantilever (nominal spring constant: 0.01 N/m) (MLCT-AUHW). Cantilevers were calibrated before and after each experiment using the thermal noise amplitude method (Hutter & Boechhoefer, 1994). Force measurements were performed using a laboratory-assembled force spectroscope optimized for vertical approach-retract cycles. An *x-y* piezo was also used in order to facilitate microscopic lateral positioning of the AFM tip over the bacteria. Force-extension curves were analyzed by Igor Pro Software (WaveMetrics, OR, USA). Unbinding forces were calculated from the vertical excursion of the last peak seen on the retraction curves.

Except for the dynamic force spectroscopy study, all approach-retract cycles were done at a velocity of 2 μm/s, with no contact time and with a low contact force. Our aim was to evaluate the fibronectin-binding frequency related to different strains or conditions. The experimental setting (force and time of contact) was then kept constant and may have resulted in multiple binding events explaining the presence of unbinding events around 2000 pN (as exemplify by Fig. 4C). The retraction velocity of 2 μm/s resulted in a mean loading rate of $2 \pm 1 \times 10^4$ pN/s, which is within the range of loading rates reported for a system mimicking the physiological situation found in blood vessels (Chen and Springer, 2001). All experiments were carried out in PBS, pH 7.3 and at a temperature of 37°C. To confirm that the data were collected over the bacteria, each approach curve was analyzed to evaluate the stiffness of the surface contacted, bacteria being softer then the glass slide (Fig. S3). Each experiment was performed at least three times using different bacteria-coated glass slides and functionalized AFM probes. No more than 50 retraction curves were usually taken from a single position. Retraction curves which did not show tip-sample interactions were considered as 0 pN unbinding events. Binding frequencies presented in unbinding forces histograms were obtained by dividing the number of unbinding events of each bin by the total number of retractions. Offsets (between 0 and 200 pN) present in unbinding force histograms reflected the probability that no or unspecific binding would be observed between the fibronectin decorated probe and the

bacteria. Mean rupture forces were only calculated on data from the 200 to 3000 pN range in order to estimate the mean unbinding force of the fibronectin-FnBPs interaction. Relative adhesions were calculated by dividing the adhesion of a particular strain or condition by the adhesion of Newbould or 8325-4 where the adhesion was defined by the sum of all unbinding forces divided by the number of retractions. The competition experiments were carried out by adding 30 nM of fibronectin or 300 nM of the recombinant fragment D1D2D3 in the AFM medium 30 min prior to the experiments. The recombinant fragment D1D2D3 was produced as described elsewhere (Gaudreau *et al.*, 2007). The bacterial contamination of the tip was evaluated by comparing the interaction of each probe on clean glass slides before and after experiments. Tips were also routinely observed during experiments with the AFM-conjugated phase contrast light microscope.

AFM-based dynamic force spectroscopy

Over 2000 unbinding forces and their related loading rates were obtained in order to generate the dynamic force spectrum of the fibronectin-FnBPs interaction at the following pulling rates: 500 nm/s (n=341), 1000 nm/s (n=129), 2000 nm/s (n=306), 5000 nm/s (n=193), 6000 nm/s (n=99), 7500 nm/s (n=98), 10000 nm/s (n=308), 12500 nm/s (n=104), 15000 nm/s (n=132), 17500 nm/s (n=123) and 20000 nm/s (n=280). The time and force of contact between the AFM probe and the bacteria were set to maximize the frequency of isolated events and to minimize the probability of multiple rupture events. For each AFM probe, bacterial culture and pulling rate, the mean unbinding force and loading rate were calculated. Results expressed in the dynamic force spectrum are the means and standard errors of the mean that had been obtained from at least three independent experiments for each pulling rate. Force measurements were corrected for hydrodynamic drag with a coefficient of 2 pN×s/µm (Wojcikiewicz *et al.*, 2006). The loading rate at which an unbinding event occurs is defined by $k_s v$ where k_s is the apparent spring constant (Fig. 1) and v is the pulling rate or the probe retraction velocity. The mean rupture forces were than plotted as a function of the logarithm of

the mean loading rates. The dynamic force spectrum curve fitting was obtained with GraphPad Prism 5.00 (GraphPad Software, CA, USA).

Briefly, there is usually a correlation between the logarithm of the loading rate and the mean unbinding force of a particular molecular interaction. This is described by the following equation (Evans and Ritchie, 1997; Mitchell *et al.*, 2007):

$$F_m = \left(\frac{k_B \times T}{\chi_B}\right) ln \left(\frac{L_r \times \chi_B}{k_{off} \times k_B \times T}\right) \quad (1)$$

where F_m is the most probable unbinding force, $k_B \times T$ is the thermal energy of the system, χ_B is the energetic barrier width or the length over which the force must be applied to reach the transition from the equilibrium position of the bound state, L_r is the loading rate and k_{off} is the off-rate. The slope of the dynamic force spectrum is $k_B \times T \div \chi_B$. The dissociation rate constant can be extrapolated to zero force in order to obtain $k_{off}(0)$ (Schwesinger *et al.*, 2000). Using equation 1, it is possible to isolate the dissociation parameters (k_{off} and χ_B) defining the energetic barrier(s) of a specific molecular interaction.

Real-time PCR.

Bacteria were collected at specific time points and treated with RNAprotect (QIAGEN). RNA was extracted from the pellets after bacterial lysis with lysostaphin (200 µg/ml during 1 h at room temperature) using the RNeasy Mini kit and the RNase-free DNase set (QIAGEN). One microgram of total RNA was reverse transcribed with 0.5 mM deoxynucleotide triphosphate,

50 ng of random hexamers and 200 U of Invitrogen Superscript II reverse transcriptase according to the manufacturer's recommendations (Invitrogen, ON, Canada). RNA was hydrolyzed and the cDNAs were purified with the QIAquick PCR purification kit (QIAGEN). One microliter of the cDNA preparation was amplified on the Stratagene MX3000P Real-Time PCR instrument with the Jump Start Taq DNA polymerase (Sigma-Aldrich), SYBR Green and 100nM of the following primers:

asp23-RT-FWD 5'-TCGCTGCACGTGAAGTTAAA-3',
asp23-RT-REV 5'-CAGCAGCTTGTTTTTCACCA-3',
fnbA268-RT-FWD 5'-ACAAGTTGAAGTGGCACAGCC-3',
fnbA341-RT-REV 5'-CCGCTACATCTGCTGATCTTGTC-3',
fnbB803-RT-FWD CACCGAAAACTGTGCAAGCA-3',
fnbB889-RT-REV 5'-TTCCTGTAGTTTCCTTATCAGCAACTT-3',
16SRNA-RT-FWD 5'- TCGTTTAACACGTTTAGGTTCA-3',
16SRNA-RT-REV 5'- GAACTGTATCAGTTGGTTTCGCAC-3',
gyrB-RT-FWD 5'-GGTGCTGGGCAAATACAAGT-3',
gyrB-RT-REV 5'-TCCCACACTAAATGGTGCAA-3'.

Reaction mixtures were denatured for 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C and 1 min 30 s at 72°C. Dissociation and standard curves were obtained to insure the specificity and the efficiency of reactions. cDNA reactions without reverse transcriptase were also routinely carried out.

The relative expression ratios in the early exponential growth phase were calculated by using the cycle threshold (C_t) of the 16S RNA of each strain as the calibrator (*n*-fold expression = $2^{-\Delta C_t}$, where ΔC_t represents the difference between the C_t of the gene studied and the C_t of the

16S RNA for each strain). In order to obtain the expression patterns of *fnbA* and *fnbB* during growth, bacteria were isolated in the early exponential, late exponential and stationary phases (Moisan *et al.*, 2006). Relative expression ratios were calculated by using the C_t of *gyrB* as the calibrator gene for each strain at each growth phase. We used *gyrB* as a calibrator during growth because *gyrB* expression was not varying significantly from the early exponential to the stationary growth phase (data not shown).

Statistical analysis.

The statistical significance of the AFM-based force spectroscopy competition experiments was evaluated by one-way analysis of variance followed by the Dunnett's multiple comparisons test. Data from experiments using the strains 8325-4 and DU5883 were analyzed with an unpaired *t* test. Statistically relevant differences between strains from the data obtained in AFM-based force spectroscopy or real-time PCR experiments (ΔC_t) were evaluated by one-way analysis of variance followed by the Bonferroni's multiple comparisons test. Statistical analyses were achieved with GraphPad Prism 5.00.

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REFERENCES

Alexander, E.H., and Hudson, M.C. (2001) Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. *Appl Microbiol Biotechnol* **56**: 361-366.

Allegrucci, M., and Sauer, K. (2007) Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *J Bacteriol* **189**: 2030-2038.

Anderson, S.W., Stapp, J.R., Burns, J.L., and Qin, X. (2007) Characterization of small-colony-variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *J Clin Microbiol* **45**: 529-535.

Archer, G.L. (1998) *Staphylococcus aureus*: a well-armed pathogen. *Clin Infect Dis* **26**: 1179-1181.

Arvidson, S., and Tegmark, K. (2001) Regulation of virulence determinants in *Staphylococcus aureus*. *Int J Med Microbiol* **291**: 159-170.

Atalla, H., Gyles, C., Jacob, C.L., Moisan, H., Malouin, F. and Mallard B. 2008. Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. *Foodborne Pathog Dis* **5**:785-99.

Bell, G.I. (1978) Models for the specific adhesion of cells to cells. *Science* **200**: 618-627.

Bischoff, M., Entenza, J.M., and Giachino, P. (2001) Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. *J Bacteriol* **183**: 5171-5179.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B., and Projan, S. (2004) Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. *J Bacteriol* **186**: 4085-4099.

Blundell, T.L., Dodson, G.G., Hodgkin, D. and Mercola, D. (1972) Insulin. The structure in the crystal and its reflection in chemistry and biology. Adv. Protein Chem. **26**: 279-402.

Bolshakova, A.V., Kiselyova, O.I., and Yaminsky, I.V. (2004) Microbial surfaces investigated using atomic force microscopy. *Biotechnol Prog* **20**: 1615-1622.

Bozzini, S., Visai, L., Pignatti, P., Petersen, T.E., and Speziale, P. (1992) Multiple binding sites in fibronectin and the staphylococcal fibronectin receptor. *Eur J Biochem* **207**: 327-333.

Brockwell, D.J., Paci, E., Zinober, R.C., Beddard, G.S., Olmsted, P.D., Smith, D.A., Perham, R.N., and Radford, S.E. (2003) Pulling geometry defines the mechanical resistance of a β-sheet protein. *Nat Struct Biol* **10**: 731-737.

Brouillette, E., Talbot, B.G., and Malouin, F. (2003) The fibronectin-binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis. *Infect Immun* **71**: 2292-2295.

Brouillette, E., Martinez, A., Boyll, B.J., Allen, N.E., and Malouin, F. (2004) Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo. *FEMS Immunol Med Microbiol* **41**: 35-41.

Bruckner, R. (1997) Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. *FEMS Microbiol Lett* **151**: 1-8.

Busscher, H.J., Norde, W., and van der Mei, H.C. (2008) Specific molecular recognition and nonspecific contributions to bacterial interaction forces. *Appl Environ Microbiol* **74**: 2559-2564.

Bustanji, Y., Arciola, C.R., Conti, M., Mandello, E., Montanaro, L., and Samori, B. (2003) Dynamics of the interaction between a fibronectin molecule and a living bacterium under mechanical force. *Proc Natl Acad Sci U S A* **100**: 13292-13297.

Chen, S., and Springer, T.A. (2001) Selectin receptor-ligand bonds: Formation limited by shear rate and dissociation governed by the Bell model. *Proc Natl Acad Sci U S A* **98**: 950-955.

Dufrene, Y.F. (2004) Using nanotechniques to explore microbial surfaces. *Nat Rev Microbiol* **2**: 451-460.

Dziewanowska, K., Patti, J.M., Deobald, C.F., Bayles, K.W., Trumble, W.R., and Bohach, G.A. (1999) Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun* **67**: 4673-4678.

Dziewanowska, K., Carson, A.R., Patti, J.M., Deobald, C.F., Bayles, K.W., and Bohach, G.A. (2000) Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells. *Infect Immun* **68**: 6321-6328.

Entenza, J.M., Moreillon, P., Senn, M.M., Kormanec, J., Dunman, P.M., Berger-Bachi, B., Projan, S., and Bischoff, M. (2005) Role of σ^B in the expression of *Staphylococcus aureus* cell wall adhesins ClfA and FnbA and contribution to infectivity in a rat model of experimental endocarditis. *Infect Immun* **73**: 990-998.

Evans, E., and Ritchie, K. (1997) Dynamic strength of molecular adhesion bonds. *Biophys J* **72**: 1541-1555.

Evans, E. (2001) Probing the relation between force--lifetime--and chemistry in single molecular bonds. *Annu Rev Biophys Biomol Struct* **30**: 105-128.

Ferrer, J.M., Lee, H., Chen, J., Pelz, B., Nakamura, F., Kamm, R.D., and Lang, M.J. (2008) Measuring molecular rupture forces between single actin filaments and actin-binding proteins. *Proc Natl Acad Sci U S A* **105**: 9221-9226.

Gaudreau, M.C., Lacasse, P., and Talbot, B.G. (2007) Protective immune responses to a multi-gene DNA vaccine against *Staphylococcus aureus*. *Vaccine* **25**: 814-824.

Greene, C., McDevitt, D., Francois, P., Vaudaux, P.E., Lew, D.P., and Foster, T.J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. *Mol Microbiol* **17**: 1143-1152.

Hamada, D., and Dobson, C.M. (2002) A kinetic study of β-lactoglobulin amyloid fibril formation promoted by urea. *Protein Sci* **11**: 2417-2426.

Haussler, S., Tummler, B., Weissbrodt, H., Rohde, M., and Steinmetz, I. (1999) Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. *Clin Infect Dis* **29**: 621-625.

Haussler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B., and Steinmetz, I. (2003) Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol* **52**: 295-301.

Hayashi, M., and Yamada, K.M. (1981) Differences in domain structures between plasma and cellular fibronectins. *J Biol Chem* **256**: 11292-11300.

Holmes, S.D., May, K., Johansson, V., Markey, F. , and Critchley, I.A. (1997) Studies on the interaction of *Staphylococcus aureus* and *Staphylococcus epidermidis* with fibronectin using surface plasmon resonance (BIAcore). *Journal of microbiological methods* **28**: 77-84.

Hutter, J.L., and Boechhoefer, J. (1994) Calibration of atomic-force microscope tips. *Rev. Sci. Instrum.* **64**: 1868-1873.

Ingham, K.C., Brew, S., Vaz, D., Sauder, D.N., and McGavin, M.J. (2004) Interaction of *Staphylococcus aureus* fibronectin-binding protein with fibronectin: affinity, stoichiometry, and modular requirements. *J Biol Chem* **279**: 42945-42953.

Inose, Y., Takeshita, S.L., Hidaka, T., Higashide, M., Maruyama, A., Hayashi, H., Morikawa, K., and Ohta, T. (2006) Genetic characterization of the natural SigB variants found in clinical isolates of *Staphylococcus aureus*. *J Gen Appl Microbiol* **52**: 259-271.

Joh, H.J., House-Pompeo, K., Patti, J.M., Gurusiddappa, S., and Hook, M. (1994) Fibronectin receptors from gram-positive bacteria: comparison of active sites. *Biochemistry* **33**: 6086-6092.

Kahl, B., Herrmann, M., Everding, A.S., Koch, H.G., Becker, K., Harms, E., Proctor, R.A., and Peters, G. (1998) Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J Infect Dis* **177**: 1023-1029.

Kahl, B.C., Becker, K., Friedrich, A.W., Clasen, J., Sinha, B., Von Eiff, C., and Peters, G. (2003) *agr*-dependent bacterial interference has no impact on long-term colonization of *Staphylococcus aureus* during persistent airway infection of cystic fibrosis patients. *J Clin Microbiol* **41**: 5199-5201.

Karlsson, A., Saravia-Otten, P., Tegmark, K., Morfeldt, E., and Arvidson, S. (2001) Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in *Staphylococcus aureus sarA* mutants due to up-regulation of extracellular proteases. *Infect Immun* **69**: 4742-4748.

Lee, C.K., Wang, Y.M., Huang, L.S., and Lin, S. (2007) Atomic force microscopy: determination of unbinding force, off rate and energy barrier for protein-ligand interaction. *Micron* **38**: 446-461.

Li, D., Renzoni, A., Estoppey, T., Bisognano, C., Francois, P., Kelley, W.L., Lew, D.P., Schrenzel, J., and Vaudaux, P. (2005) Induction of fibronectin adhesins in quinolone-resistant *Staphylococcus aureus* by subinhibitory levels of ciprofloxacin or by sigma B transcription factor activity is mediated by two separate pathways. *Antimicrob Agents Chemother* **49**: 916-924.

Liphardt, J., Onoa, B., Smith, S.B., Tinoco, I.J., and Bustamante, C. (2001) Reversible unfolding of single RNA molecules by mechanical force. *Science* **292**: 733-737.

Looney, W.J. (2000) Small-colony variants of *Staphylococcus aureus*. *Br J Biomed Sci* **57**: 317-322.

Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N Engl J Med* **339**: 520-532.

McAllister, C., Karymov, M.A., Kawano, Y., Lushnikov, A.Y., Mikheikin, A., Uversky, V.N., and Lyubchenko, Y.L. (2005) Protein interactions and misfolding analyzed by AFM force spectroscopy. *J Mol Biol* **354**: 1028-1042.

McGavin, M.J., Gurusiddappa, S., Lindgren, P.E., Lindberg, M., Raucci, G., and Hook, M. (1993) Fibronectin receptors from *Streptococcus dysgalactiae* and *Staphylococcus aureus*. Involvement of conserved residues in ligand binding. *J Biol Chem* **268**: 23946-23953.

Meenan, N.A., Visai, L., Valtulina, V., Schwarz-Linek, U., Norris, N.C., Gurusiddappa, S., Hook, M., Speziale, P., and Potts, J.R. (2007) The tandem β-zipper model defines high affinity fibronectin-binding repeats within *Staphylococcus aureus* FnBPA. *J Biol Chem* **282**: 25893-25902.

Mitchell, G., Lamontagne, C.A., Lebel, R., Grandbois, M., and Malouin, F. (2007) Single-molecule dynamic force spectroscopy of the fibronectin-heparin interaction. *Biochem Biophys Res Commun* **364**: 595-600.

Moisan, H., Brouillette, E., Jacob, C.L., Langlois-Begin, P., Michaud, S., and Malouin, F. (2006) Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *J Bacteriol* **188**: 64-76.

Nair, S.P., Bischoff, M., Senn, M.M., and Berger-Bachi, B. (2003) The σ^B regulon influences internalization of *Staphylococcus aureus* by osteoblasts. *Infect Immun* **71**: 4167-4170.

Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* **48**: 1429-1449.

Pankov, R., and Yamada, K.M. (2002) Fibronectin at a glance. *J Cell Sci* **115**: 3861-3863.

Price, C.W. (2002) General stress response. In *Bacillus subtilis and its closest relatives; from genes to cells*. Sonenshein, A.L. (ed). Washington, D.C.: ASM, pp. 369-384.

Proctor, R.A., Mosher, D.F., and Olbrantz, P.J. (1982) Fibronectin binding to *Staphylococcus aureus*. *J Biol Chem* **257**: 14788-14794.

Proctor, R.A., Balwit, J.M., and Vesga, O. (1994) Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. *Infect Agents Dis* **3**: 302-312.

Raman, E.P., Takeda, T., Barsegov, V., and Klimov, D.K. (2007) Mechanical unbinding of abeta peptides from amyloid fibrils. *J Mol Biol* **373**: 785-800.

Reddy, K., and Ross, J.M. (2001) Shear stress prevents fibronectin binding protein-mediated *Staphylococcus aureus* adhesion to resting endothelial cells. *Infect Immun* **69**: 3472-3475.

Remaut, H., and Waksman, G. (2006) Protein-protein interaction through β-strand addition. *Trends Biochem Sci* **31**: 436-444.

Sadowska, B., Bonar, A., von Eiff, C., Proctor, R.A., Chmiela, M., Rudnicka, W., and Rozalska, B. (2002) Characteristics of *Staphylococcus aureus*, isolated from airways of cystic fibrosis patients, and their small colony variants. *FEMS Immunol Med Microbiol* **32**: 191-197.

Sanderson, P.J. (1999) Orthopaedic implant infections. *Curr Opin Infect Dis* **12**: 347-350.

Saravia-Otten, P., Muller, H.P., and Arvidson, S. (1997) Transcription of *Staphylococcus aureus* fibronectin binding protein genes is negatively regulated by *agr* and an *agr*-independent mechanism. *J Bacteriol* **179**: 5259-5263.

Schwarz-Linek, U., Werner, J.M., Pickford, A.R., Gurusiddappa, S., Kim, J.H., Pilka, E.S., Briggs, J.A., Gough, T.S., Hook, M., Campbell, I.D., and Potts, J.R. (2003) Pathogenic bacteria attach to human fibronectin through a tandem β-zipper. *Nature* **423**: 177-181.

Schwesinger, F., Ros, R., Strunz, T., Anselmetti, D., Guntherodt, H.J., Honegger, A., Jermutus, L., Tiefenauer, L., and Pluckthun, A. (2000) Unbinding forces of single antibody-antigen complexes correlate with their thermal dissociation rates. *Proc Natl Acad Sci U S A* **97**: 9972-9977.

Senn, M.M., Bischoff, M., von Eiff, C., and Berger-Bachi, B. (2005) sigmaB activity in a *Staphylococcus aureus hemB* mutant. *J Bacteriol* **187**: 7397-7406.

Serpell, L.C. (2000) Alzheimer's amyloid fibrils: structure and assembly. *Biochim Biophys Acta* **1502**: 16-30.

Sompolinsky, D., Cohen, M., and Ziv, G. (1974) Epidemiological and biochemical studies on thiamine-less dwarf-colony variants of *Staphylococcus aureus* as etiological agents of bovine mastitis. *Infect Immun* **9**: 217-228.

Spearman, P., Lakey, D., Jotte, S., Chernowitz, A., Claycomb, S., and Stratton, C. (1996) Sternoclavicular joint septic arthritis with small-colony variant Staphylococcus aureus. *Diagn Microbiol Infect Dis* **26**: 13-15.

Sun, Z., Martinez-Lemus, L.A., Trache, A., Trzeciakowski, J.P., Davis, G.E., Pohl, U., and Meininger, G.A. (2005) Mechanical properties of the interaction between fibronectin and $\alpha_5\beta_1$-integrin on vascular smooth muscle cells studied using atomic force microscopy. *Am J Physiol Heart Circ Physiol* **289**: H2526-2535.

Thoumine, O., Kocian, P., Kottelat, A., and Meister, J.J. (2000) Short-term binding of fibroblasts to fibronectin: optical tweezers experiments and probabilistic analysis. *Eur Biophys J* **29**: 398-408.

Ubbink, J., and Schar-Zammaretti, P. (2005) Probing bacterial interactions: integrated approaches combining atomic force microscopy, electron microscopy and biophysical techniques. *Micron* **36**: 293-320.

Vaudaux, P., Pittet, D., Haeberli, A., Lerch, P.G., Morgenthaler, J.J., Proctor, R.A., Waldvogel, F.A., and Lew, D.P. (1993) Fibronectin is more active than fibrin or fibrinogen in promoting *Staphylococcus aureus* adherence to inserted intravascular catheters. *J Infect Dis* **167**: 633-641.

Vaudaux, P., Francois, P., Bisognano, C., Kelley, W.L., Lew, D.P., Schrenzel, J., Proctor, R.A., McNamara, P.J., Peters, G., and Von Eiff, C. (2002) Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. *Infect Immun* **70**: 5428-5437.

von Eiff, C., Peters, G., and Becker, K. (2006) The small colony variant (SCV) concept -- the role of staphylococcal SCVs in persistent infections. *Injury* **37** **Suppl 2**: S26-33.

Wojcikiewicz, E.P., Abdulreda, M.H., Zhang, X., and Moy, V.T. (2006) Force spectroscopy of LFA-1 and its ligands, ICAM-1 and ICAM-2. *Biomacromolecules* **7**: 3188-3195.

Wolz, C., Pohlmann-Dietze, P., Steinhuber, A., Chien, Y.T., Manna, A., van Wamel, W., and Cheung, A. (2000) Agr-independent regulation of fibronectin-binding protein(s) by the regulatory locus *sar* in *Staphylococcus aureus*. *Mol Microbiol* **36**: 230-243.

Xiong, Y.Q., Bayer, A.S., Yeaman, M.R., Van Wamel, W., Manna, A.C., and Cheung, A.L. (2004) Impacts of *sarA* and *agr* in *Staphylococcus aureus* strain Newman on fibronectin-binding protein A gene expression and fibronectin adherence capacity in vitro and in experimental infective endocarditis. *Infect Immun* **72**: 1832-1836.

Yip, C.M., Yip, C.C., and Ward, M.D. (1998) Direct force measurements of insulin monomer-monomer interactions. *Biochemistry* **37**: 5439-5449.

Yongsunthon, R., Fowler, V.G., Jr., Lower, B.H., Vellano, F.P., 3rd, Alexander, E., Reller, L.B., Corey, G.R., and Lower, S.K. (2007) Correlation between fundamental binding forces and clinical prognosis of *Staphylococcus aureus* infections of medical implants. *Langmuir* **23**: 2289-2292.

Zierdt, C.H., and Schmidt, P.J. (1964) Dissociation in *Pseudomonas aeruginosa*. *J Bacteriol* **87**: 1003-1010.

Zlatanova, J., Lindsay, S.M., and Leuba, S.H. (2000) Single molecule force spectroscopy in biology using the atomic force microscope. *Prog Biophys Mol Biol* **74**: 37-61.

CHAPITRE 2

A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides

2.1. Introduction de l'article

L'objectif de cette étude a été de déterminer *in vitro* et *in vivo* le rôle de SigB dans la formation de SCVs et de biofilm lors d'une exposition aux aminoglycosides, une classe d'antibiotiques utilisée dans le traitement des patients FK. L'étudiant David Lalonde Séguin et moi avons effectué l'ensemble des expériences de microbiologie à l'aide de la stagiaire Ann-Élise Asselin. J'ai assisté le professionnel de recherche Éric Brouillette lors des expériences utilisant le modèle d'infection de la mammite murine. L'étudiant Christian Lebeau Jacob et moi avons effectué les PCR quantitatives en temps réel. J'ai rédigé l'ébauche de l'article, effectué la conception des figures et participé à la correction du manuscrit jusqu'à sa publication.

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2.2. Résumé en français de l'article

Les variants à petites colonies (SCVs) et les biofilms de *Staphylococcus aureus* sont associés aux infections chroniques. Il est connu que la présence d'antibiotiques de la classe des aminoglycosides peut contribuer à l'émergence de SCVs et on assume que des mécanismes moléculaires sont impliqués dans l'habileté qu'a *S. aureus* d'adopter ce phénotype. Aucune étude n'a encore adressé le possible rôle du facteur sigma alternatif sigma B (SigB) dans l'émergence des SCVs, même si une activité constitutive de SigB a été rapportée chez ces souches. Ici, nous démontrons que SigB est impliqué dans l'émergence de SCVs lors d'expositions à une concentration sous-inhibitrice d'aminoglycosides. Le suivi de l'expression de gènes chez une souche prototypique traitée aux aminoglycosides ou chez des SCVs cliniques a montré l'activation de SigB tandis que le système *agr* ne l'était pas. En outre, les bactéries prototypiques traitées à la gentamicine et les SCVs avaient une habileté augmentée à former du biofilm seulement dans un contexte où SigB était fonctionnel. L'administration d'une concentration sous-inhibitrice de gentamicine augmentait significativement la formation de SCVs pour une souche prototypique, mais par pour un mutant *sigB*, dans un modèle murin de mammite induite à *S. aureus*. Collectivement, nos résultats montrent que SigB peut influencer positivement l'émergence de SCVs et la production d'un biofilm suite à une exposition de *S. aureus* aux aminoglycosides.

2.3. L'article scientifique

A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides

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Keywords: mastitis, cystic fibrosis, chronic infections, SarA, SigB, *agr*.

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ABSTRACT

Staphylococcus aureus small-colony variants (SCVs) and biofilms are linked to chronic infections. It is known that the presence of aminoglycoside antibiotics may contribute to the emergence of SCVs and it is thought that molecular mechanisms are involved in the ability of *S. aureus* to adopt this phenotype. No study has addressed the possible role of the stress- and colonization-related alternative sigma factor B (SigB) in the emergence of SCVs, although a sustained SigB activity was reported in these variants. Here, we demonstrate that SigB is involved in the emergence of SCVs resulting from an exposure to a sub-inhibitory concentration of aminoglycosides. Monitoring of gene expression in an aminoglycoside-treated prototypical strain or in clinical SCVs showed the activation of SigB, whereas the accessory gene regulator (*agr*) system was not. Furthermore, gentamicin-treated prototypical bacteria and SCVs had an increased ability to form biofilm only in a SigB functional background. The administration of a sub-inhibitory concentration of gentamicin significantly increased the formation of SCVs for a prototypical strain but not for the *sigB* mutant in a mouse model of *S. aureus*-induced mastitis. Collectively, our results show that SigB may positively influence the appearance of *S. aureus* SCVs and the production of biofilm upon aminoglycoside exposure.

1. INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen which can cause multiple types of serious infections with high morbidity rates but also persistent and recurring infections. The ability of *S. aureus* to cause diverse diseases has been linked to the numerous virulence factors which allow the bacterium to evade host defenses, to adhere to or destruct host tissues and to induce sepsis syndromes [1]. Diverse and complex regulatory networks control the expression of these virulence factors in a time- and environment-dependent manner.

It is thought that the transition from the colonization to a dissemination phase during infection is controlled by the accessory gene regulator (*agr*) system [2, 3], whereas stress responses and adhesin expression needed for host tissues colonization are positively influenced by the activity of the alternative transcription factor sigma B (SigB) [4-7]. During the exponential growth of prototypical *S. aureus* strains *in vitro*, the transcription of the *hld* gene which encodes RNAIII, a regulatory RNA, is induced through the *agr* quorum sensing dependent two-component system. The *agr* system impacts on the expression of several exotoxins (*e.g.*, α-hemolysin) and proteolytic enzymes, and represses the expression of cell-surface proteins involved in colonization [2]. On the other hand, SigB plays an important role in regulating genes in response to environmental stresses, as for example, during stationary phase, heat exposure and change in osmotic pressure (for review see [8]). An important consequence of SigB activity is the up-regulation in expression of a multitude of cell-surface proteins such as the fibronectin-binding protein A (FnBP A) and the down-regulation of a variety of exotoxins [4, 9]. The genes regulated by SigB also include another global regulator of virulence, *sarA* (staphylococcal accessory regulator) [4, 9]. SarA modulates the expression of several virulence factors either by stimulating RNAIII transcription or by pathway(s) independent of the *agr* system. Overall, the *sarA* locus affects at least 120 genes in *S. aureus*, up-regulating the expression of hemolysins as well as the FnBPs [10]. To add further complexity, there is a

possible interplay between SigB and *agr* since mutants in the *sigB* operon show an altered *agr* activity [11].

Previous studies have demonstrated that the formation of biofilm by *S. aureus* can be influenced by *agr* [12, 13], SigB [12-15] and SarA [13, 16]. It has been shown that bacterial biofilm may confer protection from host defenses as well as tolerance to at least some antibiotics, biocides and hydrodynamic shear forces [17]. The intrinsic resistance of biofilm-associated bacteria to antibiotics may be explained by a reduced growth rate that alters their susceptibility to antibiotics that target cell wall biosynthesis whereas a reduced oxidative metabolism impedes uptake of aminoglycoside antibiotics. It was estimated that biofilm formation is involved in 65% of nosocomial infections in the United States [18] and most of chronic infections [19, 20]. More particularly, *S. aureus* intra-mammary infections often lead to chronic and difficult-to-treat bovine mastitis [21-23], and genomic loci involved in biofilm formation are found in most clinical bovine mastitis strains [24, 25]. Biofilm formation also appears to play a role in lung infections persisting in cystic fibrosis (CF) patients [26].

S. aureus small-colony variants (SCVs) are often described as a sub-population of slow-growing respiratory deficient bacteria that are auxotroph for hemin or menadione with a reduced susceptibility to aminoglycosides [27]. Interestingly, SCVs are frequently isolated from chronic infections [27, 28] as in the case of infections of the airways of CF patients [7, 29-31] and infections of the bovine mammary gland during mastitis [30, 32]. Several studies have shown that *S. aureus* SCVs possess an increased capacity to invade and persist in host cells [7, 29, 33], which may help the establishment of chronic infections by offering the bacterium a protection against the immune system and the action of antibiotics [33, 34]. Using SCVs isolated from cystic fibrosis patients, we have previously demonstrated that SigB activity influences the transcription of several virulence factors and is associated with an increased capacity to invade and persist within host cells [7]. In addition, we have also

recently shown that CF-isolated SCVs are locked into a colonization state as a result of a sustained SigB activity and expression of genes encoding cell-surface proteins [6, 7]. Interestingly, many biofilm-associated genes are transcribed in SCVs isolated from cystic fibrosis patients [7] and the SCV phenotype was recently associated with an increased in biofilm formation [35].

It has been shown that some environmental factors such as the mammalian intracellular milieu [36] and the presence of aminogycosides such as gentamicin and tobramycin [27, 37-40] may contribute to the emergence of the SCV phenotype. Noteworthy, aminoglycosides are often used for treatment of infections in CF patients [26] and have also been used to treat bovine mastitis [41, 42]. It is thought that the ability of *S. aureus* to switch between the SCV and the prototypical wild-type phenotype may involve underlying regulatory and/or genetic mechanisms [38]. Although results from Schaaff *et al.* [39] suggest that mutations play a role in the development of SCVs, experiments were performed in the *S. aureus* strain 8325-4, which is a strain characterized by a lower SigB activity resulting from a "natural" deletion in the *rsbU* locus [43]. Therefore, questions about the possible influence of the alternative sigma factor B on the emergence of the SCV phenotype are still unanswered at this time [27, 39, 44].

The aim of this work was to investigate the possible role played by SigB in the emergence of the SCV phenotype upon an exposure to aminoglycosides. We found that the activity of the SigB contributes to the emergence of SCVs resulting from an exposure to aminoglycosides by comparing the quantity of SCVs generated by a prototypical strain and its *sigB* mutant. While SigB-dependent genes were up-regulated in both SCVs isolated from cystic fibrosis patients and from bovine mastitis, and also in prototypical bacteria exposed *in vitro* to aminoglycosides, the *agr* system was not activated. Interestingly, the observed modulations in gene expression were consistent with a SigB-dependent increased in biofilm formation in the clinical SCV isolates as well as in the gentamicin-treated prototypical bacteria. A role for SigB

in the emergence of the SCV phenotype in response of prototypical bacteria to an aminoglycoside treatment was also established *in vivo* in a mouse model of *S. aureus*-induced mastitis.

2. RESULTS

2.1. SigB is involved in the emergence of SCVs resulting from an exposure to a sub-inhibitory concentration of aminoglycosides. In order to investigate the role of SigB in the emergence of the SCV phenotype upon exposure to aminoglycosides, strains Newbould and NewbouldΔ*sigB* were exposed to sub-inhibitory concentrations of antibiotics and the frequency of SCVs was determined. NewbouldΔ*sigB* is a *sigB* knock-out isogenic to the prototypical strain Newbould which has already been used before [6, 7]. The pictures from Fig. 1A illustrate the ability of a sub-inhibitory concentration (0.5 × MIC) of gentamicin to favor the emergence of the SCV phenotype in a *S. aureus* $sigB^+$ background only. Fig. 1B shows that a sub-inhibitory concentration of gentamicin significantly increased the presence of SCVs generated from strain Newbould (283 fold, $P < 0.01$). The ability of gentamicin to quantitatively increase the number of SCVs in *S. aureus* populations was also seen with strains of other genetic backgrounds such as ATCC 29213, SH1000 and N315 as well as with three non-SCV strains isolated from CF patients and one from bovine mastitis (strain 3231described in the methods section) (data not shown). Another aminoglycoside, tobramycin, also promoted the emergence of the SCV phenotype in strain Newbould (42 fold, $P < 0.05$, Fig. 1B) as well as in strain ATCC 29213 (data no shown). Besides, no significant increase in SCVs was observed with NewbouldΔ*sigB* in the presence of gentamicin when compared to the untreated condition and significantly fewer SCVs were recovered from NewbouldΔ*sigB* in comparison to Newbould in presence of a sub-inhibitory concentration of gentamicin ($P < 0.01$). A role for SigB in the emergence of SCVs resulting from an exposure to gentamicin was also

demonstrated with strains SH1000 and 8325-4 (data not shown), which are isogenic strains with a functional and dysfunctional *rsbU* gene that impacts on SigB activity, respectively.

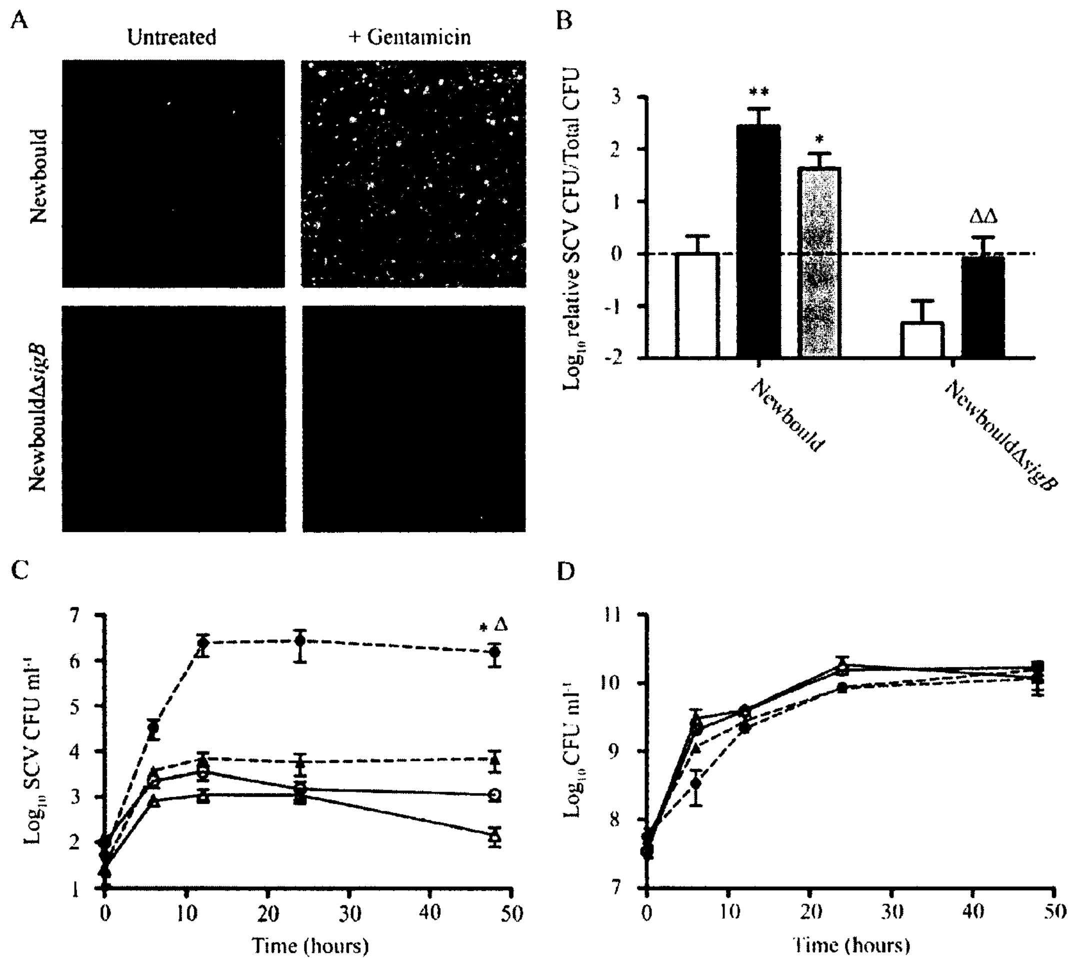

FIGURE 1. SigB is involved in the emergence of SCVs resulting from an exposure to a sub-inhibitory concentration of aminoglycosides. (A) Pictures show SCV colonies grown on agar containing a selective concentration of gentamicin following or not an overnight treatment of strains Newbould and NewbouldΔ*sigB* with a sub-inhibitory concentration of gentamicin. (B) Relative number of SCV CFU recovered after 18 h of growth for strains Newbould and

NewbouldΔ*sigB* following (black or grey bars for gentamicin or tobramycin, respectively) or not (open bars) an overnight treatment with a sub-inhibitory concentration of antibiotic. Results are normalized relatively to untreated Newbould. Data are presented as means with standard deviations from at least three independent experiments. Kinetics of SCV formation (C) and growth curves (D) for Newbould (●) and NewbouldΔ*sigB* (▲) exposed (black symbols, dotted lines) or not (open symbols, solid lines) to a sub-inhibitory concentration of gentamicin for the indicated time. Data are presented as means with the standard errors of the mean from three independent experiments. Significant differences between untreated and antibiotic-treated conditions (*, $P<0.05$; **, $P<0.01$; unpaired *t*-test) and between Newbould and NewbouldΔ*sigB* for same experimental conditions (Δ, $P<0.05$, $\Delta\Delta$, $P<0.01$; ANOVA with Tuckey's post test) are shown.

The kinetics of SCV formation was also studied. SCV formation from Newbould and NewbouldΔ*sigB*, exposed or not to gentamicin, was monitored as a function of time (Fig. 1C). The SCV sub-population from strain Newbould was quickly and significantly increased by more than 1000 times in the presence of gentamicin ($P < 0.05$), in contrast to that observed with NewbouldΔ*sigB* or any of the two strains in the absence of gentamicin. Notheworthy, the growth curves of Newbould and NewbouldΔ*sigB* with and without gentamicin at $0.5 \times$ MIC were not significantly different (Fig. 1D), suggesting that the differences in the kinetics of SCV formation observed in Fig. 1C were not due to differences in growth. These results suggest that SigB supports the emergence of SCVs resulting from an exposure to a sub-inhibitory concentration of aminoglycosides.

2.2. SigB and *agr* activities are modulated by exposure to a sub-inhibitory concentration of aminoglycosides. We performed transcriptional analyses of key genes in order to verify the hypothesis that SigB is activated during exposure to aminoglycosides. Fig. 2 shows qPCR measurements of the expression of *asp23, fnbA, hld* (RNAIII), *hla*, *sarA* and *gyrB* in

Newbould and NewbouldΔ*sigB* strains exposed or not to a sub-inhibitory concentration of aminoglycosides during exponential growth. The gene *asp23* is a well-known marker for SigB activity as for the gene *fnbA*, although the transcription of the latter is not exclusively influenced by SigB [4-7, 45]. The expression level of these genes was used instead of the expression of *sigB* itself because the regulation of the activity of SigB involves multiple protein-protein interactions rather than only transcriptional regulation [8]. Fig. 2A and 2B show that a sub-inhibitory concentration of gentamicin and tobramycin induced SigB activity in *S. aureus* Newbould. The expression of *asp23* (Fig. 2A) was significantly induced by both gentamicin (3.7 fold, $P < 0.05$) and tobramycin (7.4 fold, $P < 0.001$) as was the expression of *fnbA* (Fig. 2B) (25.7 fold [$P < 0.01$] and 35.5 fold [$P < 0.05$], respectively). These results also show a significant down-regulation of *asp23* in both untreated and gentamicin-treated NewbouldΔ*sigB* in comparison to Newbould ($P < 0.001$ for both comparisons). Similarly, the expression of *fnbA* in Newbould treated with gentamicin was significantly higher than that of NewbouldΔ*sigB* under the same condition ($P < 0.05$). The effect of gentamicin on the expression of *asp23* and *fnbA* was also observed in strains of other genetic backgrounds such as the sequenced strain Newman as well as in an isolate from bovine mastitis (strain 3231) (data not shown). This confirms the role of SigB in the up-regulation of *asp23* and *fnbA* expression under exposure to aminoglycosides.

The activity of the *agr* system was also monitored since it is known to be reduced in SCVs [7, 32, 46, 47]. Fig. 2C shows that a sub-inhibitory concentration of gentamicin significantly repressed the expression of *hld* (the effector of the *agr* system) in strain Newbould (13.7 fold, $P < 0.01$) although the observed reduction with the tobramycin treatment was not statistically significant. However, the expression of *hla* was significantly repressed by both treatments (respectively 2.3 fold, $P < 0.01$ and 2.9 fold, $P < 0.01$) in strain Newbould (Fig. 2D) and the expression of *hld* and *hla* was also decreased by gentamicin treatment in NewbouldΔ*sigB*, although not significantly (Fig. 2C and 2D). Furthermore, the expression of *hla* was, in both untreated and gentamicin-treated conditions, significantly increased in NewbouldΔ*sigB* in

comparison to Newbould ($P < 0.05$ for both conditions), which confirms the negative influence of SigB on *hla* expression [43]. The effect of gentamicin on the expression of *hld* was also confirmed in strain Newman and in an isolate from bovine mastitis (strain 3231) (data not shown). These transcriptional results show that the activation of the *agr* system can be reduced by a sub-inhibitory concentration of aminoglycosides.

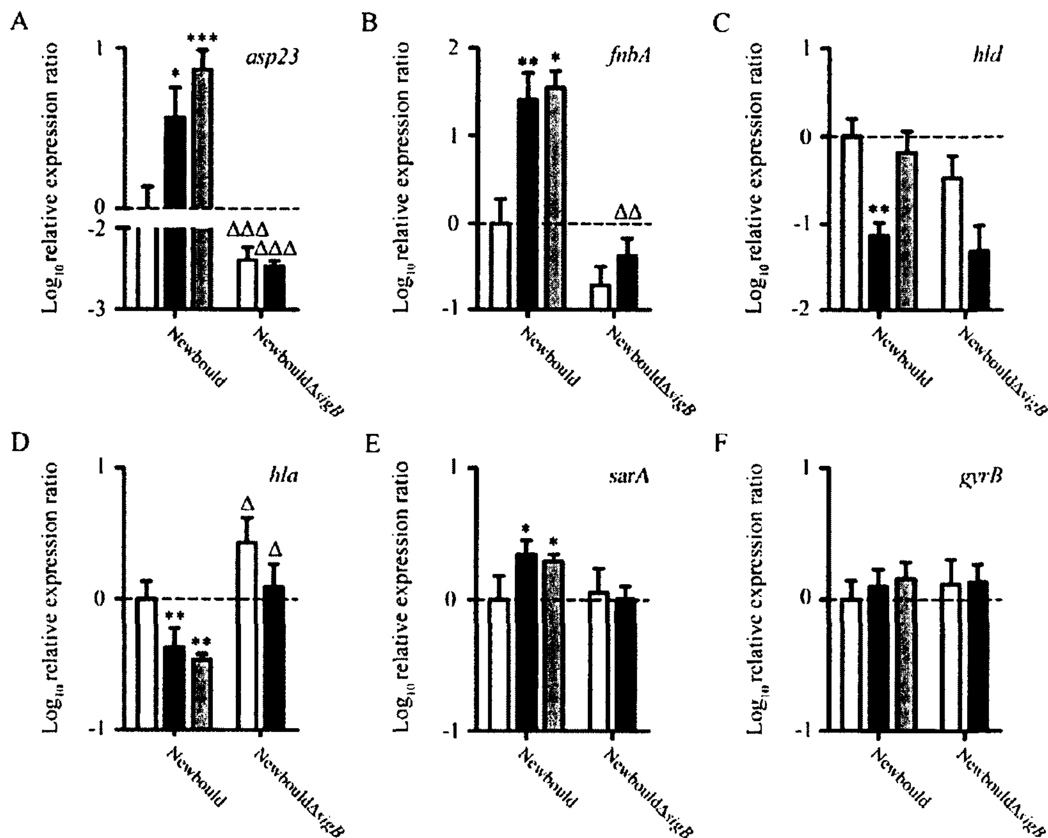

FIGURE 2. SigB and *agr* activities are modulated by exposure to a sub-inhibitory concentration of aminoglycosides. Relative expression ratios for the genes *asp23* (A), *fnbA* (B), *hld* (C), *hla* (D), *sarA* (E) and *gyrB* (F) were evaluated by qPCR for strains Newbould and NewbouldΔ*sigB* grown to the exponential phase in the presence (black or grey bars for

gentamicin or tobramycin, respectively) or in the absence (open bars) of a sub-inhibitory concentration of antibiotic. Results are normalized to untreated Newbould. Data are presented as means with standard deviations from three independent experiments (the exception was in B, where the assay with tobramycin was done twice). Significant differences between untreated and antibiotic-treated conditions (*, $P<0.05$;**, $P<0.01$; ***, $P<0.001$; ANOVA with Dunnett's post test and unpaired *t*-test for Newbould and NewbouldΔ*sigB*, respectively) and between Newbould and NewbouldΔ*sigB* for same experimental conditions (Δ, $P<0.01$; $\Delta\Delta$, $P<0.01$; $\Delta\Delta\Delta$, $P<0.001$; ANOVA with Tuckey's post test) are shown.

The expression level of *sarA* was also measured because of its partial dependency on SigB [4, 9] as well as for its role in the regulation of expression of virulence factors [10]. Of striking interest, *sarA* expression was significantly induced by gentamicin and tobramycin in strain Newbould (respectively 2.2 fold, $P < 0.05$ and 1.9 fold, $P < 0.05$) whereas this phenomenon was not observed with NewbouldΔ*sigB* (Fig. 2E).The amplitude, although small, of this SigB-dependent increase in *sarA* expression is in the range of values also obtained by Bischoff et *al.* (2004) when comparing *sarA* expression of different bacterial strains with their respective *sigB* operon mutants. Besides, Fig. 2F shows that aminoglycoside treatments had no impact on the expression of the housekeeping gene *gyrB* in Newbould or in NewbouldΔ*sigB*, further validating the observed modulations of *sarA* expression. These results suggest that a sub-inhibitory concentration of aminoglycoside induces the expression of *sarA* only in a SigB-positive background.

2.3. Status of SigB, *agr* and *sarA* in clinical SCVs isolated from cystic fibrosis patients and bovine mastitis. Since SigB is apparently needed for the emergence of SCVs and that *sarA* expression is only induced in a SigB-positive background upon exposure to aminoglycosides, it is relevant to also evaluate SigB and *agr* activities in preformed, naturally-occurring, clinical SCVs. We indeed previously showed that clinical SCVs have a

transcriptional signature of their own and that many genes under the positive control of SigB are specifically up-regulated in comparison to that seen in prototypical strains [7]. In order to further confirm that the activities of SigB and *agr* as well as the expression of *sarA* are specifically modulated in naturally-occurring clinical SCVs, we conducted qPCR experiments with strains CF07 and CF07Δ*sigB* in the early exponential phase of growth. Since CF07 is a menadione-auxotroph clinical SCV [7], we were able to use a chemical complementation of the SCV phenotype to compare SigB, *agr* and *sarA* status in an isogenic background (CF07 *vs.* CF07 with menadione) in addition to the comparison of CF07 to CF07Δ*sigB*. Fig. 3A shows that CF07 can indeed achieve a growth rate comparable to that of the non-SCV prototypical strain Newbould when grown in presence of menadione. For this part of the study, we also used an additional pair of naturally-occurring SCV and non-SCV isogenic strains (Heba3231 and 3231, respectively) which were previously confirmed as derivatives of the same clone isolated from a case of chronic bovine mastitis [32].

Fig. 3B shows the expression level of *asp23* (marker of SigB activity) in the studied strains. The SigB activity of CF07 was found to be significantly reduced by supplemental menadione according to *asp23* expression ($P < 0.01$). The SigB-dependency for expression was also once again validated by the significant repression in CF07Δ*sigB* in comparison to CF07 ($P < 0.001$). Also, the expression level of *asp23* was significantly lower in strain 3231 than that observed in its isogenic counterpart SCV Heba3231 (5.0 fold, $P < 0.01$). These results confirm that the activity of SigB is increased in at least two different SCV backgrounds comparatively to non-SCV strains.

Fig. 3C shows the expression level of *hld* in the studied strains. The expression level of *hld* was significantly up-regulated when the growth of the SCV CF07 was complemented by the presence of menadione ($P < 0.05$). The inactivation of *sigB* in CF07 also resulted in a significant up-regulation of the expression of *hld* ($P < 0.001$), which confirmed the interplay

between the *agr* system and SigB [11]. Noteworthy, the hemolysis caused by CF07Δ*sigB* is indeed notably higher than that of CF07 when grown overnight on agar plates containing 5% of sheep blood (data not shown). The expression of *hld* was also higher in strain Heba3231 in comparison to the isogenic SCV Heba3231 (33.7 fold, $P < 0.001$). These results confirm that the *agr* system is relatively not activated in SCVs.

The expression level of *sarA* in the studied strains is shown in Fig. 3D. As for *asp23*, the expression level of *sarA* was repressed by the presence of menadione in the growth medium ($P < 0.01$). Furthermore, the expression of *sarA* was down-regulated in CF07Δ*sigB* in comparison to CF07 ($P < 0.01$), which confirmed the previously shown influence of SigB on *sarA* expression [4, 9]. The up-regulation of *sarA* expression in SCVs was also confirmed with the SCV and non-SCV 3231 pair (Fig. 3D). Strain 3231 was indeed expressing *sarA* significantly less than the SCV Heba3231 (3.7 fold, $P<0.05$). These results suggest that *sarA* is specifically up-regulated in SCVs, possibly through a SigB-dependent mechanism. Overall, these results suggest that SigB activation and *agr* repression specifically occur in naturally-occurring SCVs isolated from either CF patients or bovine mastitis. This cellular status is thus likely to modulate the expression of virulence factors and the pathogenesis of SCVs differently than that normally occurring in prototypical *S. aureus*.

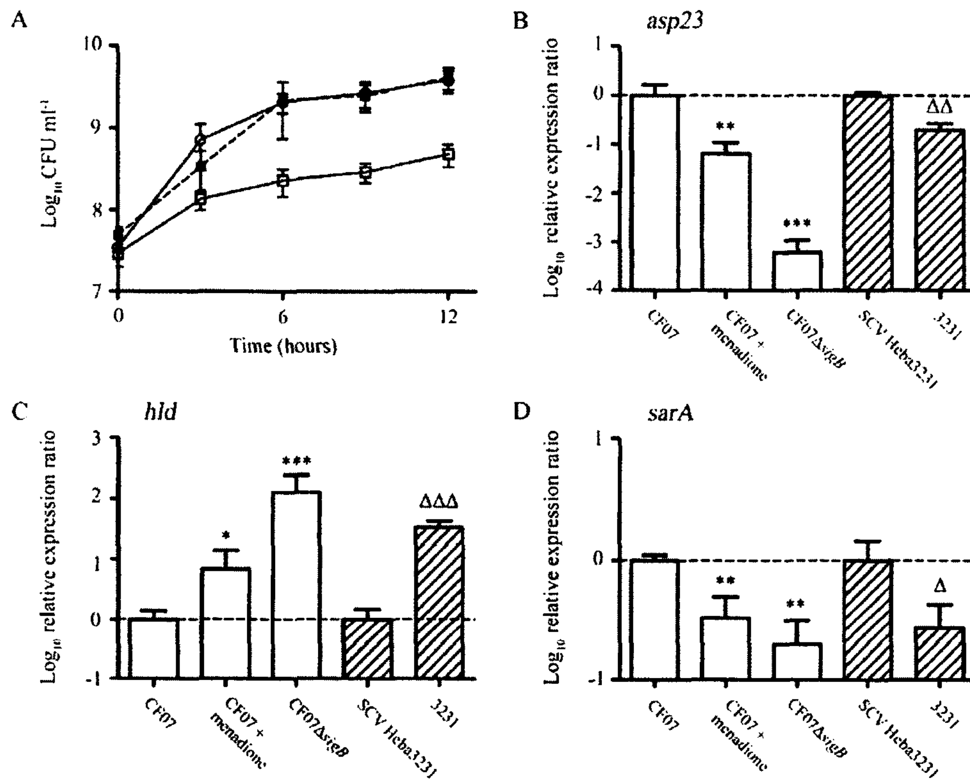

FIGURE 3. Status of SigB, *agr* and *sarA* in clinical SCVs isolated from cystic fibrosis patients and bovine mastitis. (A) Growth curves for the non-SCV strain Newbould (open circles) and the menadione-auxotroph SCV CF07, with (black squares, dotted line) and without (open squares, solid line) menadione in the growth medium. Relative expression ratios for the genes *asp23* (B), *hld* (C) and *sarA* (D) were evaluated by qPCR for strains CF07, CF07 with menadione, CF07Δ*sigB*, SCV Heba3231 and 3231, in the early exponential phase of growth. Results are normalized to the SCV CF07 (open bars) or SCV Heba3231 (hatched bars). Data are presented as means with standard deviations from at least three independent experiments. Significant differences in comparison to CF07 (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ANOVA with Dunnett's post test) or SCV Heba3231 (Δ, $P<0.05$; ΔΔ, $P<0.01$; ΔΔΔ, $P<0.001$; unpaired t-test) are shown.

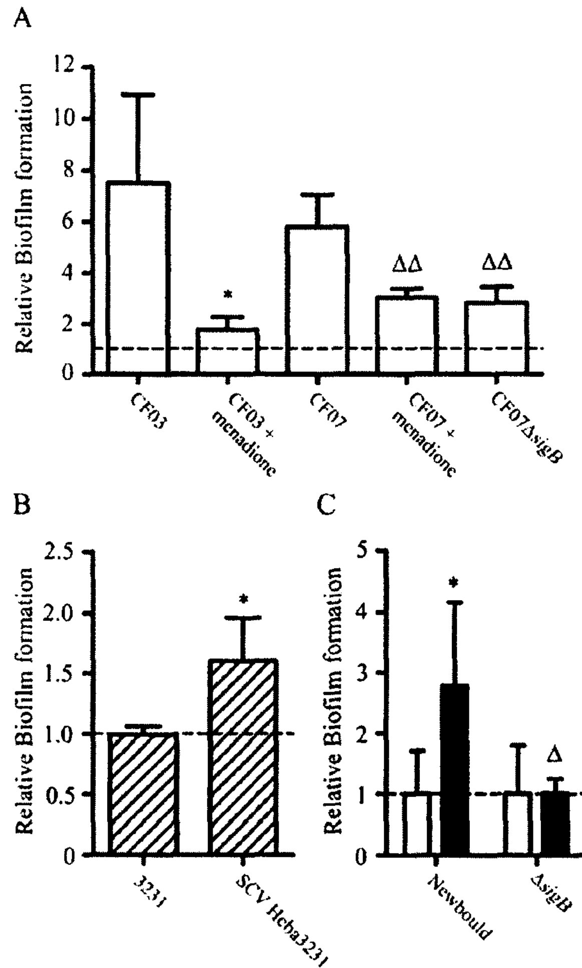

FIGURE 4. SigB is involved in biofilm formation of clinical SCVs and gentamicin-treated prototypical bacteria. (A) Relative biofilm formation of strains CF03, CF03 with menadione, CF07, CF07 with menadione and CF07Δ*sigB*. Significant differences in comparison to CF03 (*, $P<0.05$; unpaired *t*-test) and CF07 (Δ, $P<0.05$; ΔΔ, $P<0.01$; ANOVA with Dunnett's post test) are shown. (B) Relative biofilm formation of 3231 and SCV Heba 3231. A significant difference between 3231 and SCV Heba3231 (*, $P<0.05$; unpaired *t*-test) is shown. (C) Relative biofilm formation of Newbould and NewbouldΔ*sigB* exposed or not to a sub-inhibitory concentration of gentamicin. Significant differences in comparison to untreated Newbould (*, $P<0.05$; unpaired *t*-test) or between Newbould and NewbouldΔ*sigB* exposed to

gentamicin (Δ, $P<0.05$; ANOVA with Tuckey's post test) are shown. All biofilm results are normalized to the untreated Newbould strain (dotted line). Data are presented as means with standard deviations from at least three independent experiments.

2.4. SigB is involved in biofilm formation of clinical SCVs and gentamicin-treated bacteria. According to the relative role of SigB, SarA and *agr* on the formation of *S. aureus* biofilm, we hypothesized that SCVs as well as gentamicin-treated prototypical bacteria should demonstrate an increased ability to form biofilm. Fig. 4A shows that two clinical menadione-auxotroph SCVs isolated from CF patients formed significantly more biofilm than these same stains under metabolic complementation using supplemental menadione ($P < 0.05$ and $P < 0.01$ for CF03 and CF07, respectively). The use of supplemental menadione did not alter the biofilm formation of three non-SCV strains (data not shown). Interestingly, the biofilm formation of CF07 was significantly reduced in the *sigB* mutant ($P < 0.01$). The increased biofilm formation of SCVs in comparison to the non-SCV strains was also demonstrated using the clonal derivatives 3231 and SCV Heba3231 in Fig. 4B (1.6 fold, $P < 0.05$). Fig. 4C shows that exposure of Newbould to a sub-inhibitory concentration of gentamicin significantly increased biofilm formation (2.8 fold, $P < 0.05$), whereas the antibiotic could not further increase biofilm formation in NewbouldΔ*sigB*. The effect of gentamicin on biofilm formation was also observed in other genetic backgrounds with strain Newman and with a non-SCV strain isolated from a CF patient (data not shown). These results suggest that SCVs and gentamicin-treated prototypical *S. aureus* have an increased ability to form biofilm. Results with the two Δ*sigB* mutants suggest that this ability is mediated trough a SigB-dependent mechanism.

2.5. SigB is involved in the emergence of SCVs resulting from gentamicin exposure *in vivo*. We used a mouse model of *S. aureus*-induced mastitis in order to confirm the relevance of our findings *in vivo*. Strain Newbould was chosen for these experiments because it was

originally isolated from a case of bovine mastitis [48] and is thus more relevant for this infection model. Gentamicin was chosen as the test aminoglycoside because it has been used to treat bovine mastitis [41, 42]. Gentamicin was administered intravenously twice during the course of the infection and the emergence of the SCV phenotype was evaluated after 18 h. Several concentrations of gentamicin were tested in preliminary experiments (data not shown) and a concentration that was sub-inhibitory for *S. aureus* colonization of the mammary gland but sufficiently high to promote the emergence of SCVs was selected. Fig. 5A shows that neither the concentration of gentamicin used nor the knock-out of *sigB* in the Newbould background altered the number of CFU recovered from the mammary glands 18 h post-infection. This is suggesting that SigB does not significantly contribute to the short-term colonization of the mouse mammary glands or at least in the absence of a suckling pressure (or milking by analogy to the cow). However, the administration of a sub-inhibitory concentration of gentamicin significantly increased the number of SCVs isolated from the infected glands for the Newbould strain ($P < 0.05$) but not for NewbouldΔ*sigB* (Fig. 5B). The median number of SCVs recovered from the mammary glands infected by Newbould was increased by about 49 fold when mice were treated with a sub-inhibitory concentration of gentamicin. These observations support the hypothesis that treatment of *S. aureus* infections with aminoglycosides can favor the emergence of SCVs and that SigB is involved in this phenomenon *in vivo*.

A

B

No of glands: 8 8 10 10

FIGURE 5. SigB is involved in the emergence of SCVs resulting from gentamicin exposure *in vivo*. For each lactating mouse, the R4 and L4 mammary glands were infected with Newbould and NewbouldΔ*sigB*, respectively. Two doses of gentamicin (0.5 mg kg^{-1}) were provided by intravenous administration at 2h and 10 h post-infection. Medians values (dotted bars) of CFU (A) and SCV CFU (B) per gram of gland respectively recovered on TSA plates and on selective gentamicin plates are indicated. The detection limit (d.l.) was of 25 SCV CFU per gland. The statistical difference between SCV CFU recovered from strain Newbould in treated and untreated animals is shown (non-parametric Kruskal-Wallis ANOVA with Dunn's post test). The number of glands used in both A and B is indicated at the bottom of panel.

3. DISCUSSION

The association between the SCV phenotype and persistent infections is now generally recognized (for review see [27]). SCVs are thought to be a subpopulation of bacteria that can hide from the immune system and antibiotics by invading or being internalized by host cells. This, combined to the slow growth rate and the ability to revert to the wild-type phenotype, may explain the difficulty encountered in treating some staphylococcal infections and why recurrence sometimes happen days or months after apparently successful treatments. Importantly, SCVs have previously been recovered from patients who had been treated with gentamicin beads [40] and an association between aminoglycoside therapy and persistent SCV has been proposed [29]. The present study confirms the *in vitro* observation that gentamicin and tobramycin favor the emergence of the SCV phenotype and demonstrates for the first time that systemic administration of gentamicin can indeed provoke the emergence of SCVs *in vivo.*

It has indeed been known for quite some time that *in vitro* exposure of *S. aureus* to gentamicin results in the emergence of aminoglycoside-resistant variants which are non-hemolytic [49] and that gentamicin inhibits the production of hemolysins by *S. aureus* [50]. Our results suggest that this decreased production of hemolysins is probably caused by the repression of the *agr* system and/or the induction of SigB activity. Interestingly, sub-inhibitory concentrations of gentamicin induce the expression of genes associated with the heat shock response in *Bacillus subtilis* [51], which has been related to SigB in *B. subtilis* as well as in *S. aureus* [8]. Our results showed that aminoglycosides affect the activity of SigB and *agr* in *S. aureus* and also suggest that aminoglycosides stimulate the formation of biofilm by influencing SigB, *agr* and SarA.

At least two different mechanisms of biofilm formation exist in *S. aureus* [16, 52, 53]. The first mechanism implies the production of the polysaccharide intercellular adhesin (PAI), which requires the *ica* gene cluster, whereas the second mechanism is *ica*-independent. It has been shown that SarA and *agr* are both involved in the *ica*-independent mechanism of biofilm formation, SarA being required for the initial attachment step while *agr* is controlling the dispersal process [12, 16, 52]. Lauderdale *et al.* [13] has recently shown that SigB is also an essential regulator of the *ica*-independent mechanism of biofilm formation and suggests that SigB acts upstream of the *agr* system, allowing the biofilm formation to be regulated as a function of environmental factors. We observed no significant effects on the expression of *icaC* and *icaB* when Newbould was treated with a sub-inhibitory concentration of either gentamicin or tobramycin (data not shown). Collectively, our results thus suggest that gentamicin stimulates *S. aureus* to form biofilm by the *ica*-independent mechanism and confirm the predominant role of SigB in this mechanism.

The *agr* system was shown not to be activated in SCVs [7, 32, 46, 47] while SigB activity appeared constitutive [6, 7, 45]. The SCVs studied here also demonstrated a decreased *agr* and an increased SigB activity but also an up-regulated expression of *sarA* in comparison to non-SCV strains. This was in accordance to their increased ability to form biofilm, possibly through a SigB-dependent mechanism. The idea of an *ica*-independent mechanism of biofilm formation in SCVs was also supported by the demonstration that the expression of *icaC* and *icaB* genes was not influenced by the metabolic complementation of CF07 with menadione (data not shown). We have recently shown that the constitutive SigB activity in SCVs isolated from cystic fibrosis locks the ability of *S. aureus* to strongly bind fibronectin during growth by sustaining the expression of *fnbA* [6]. Because of the also recent demonstration of the involvement of FnBPs in the *ica*-independent mechanism of biofilm formation [52] and because of the low activity of the *agr* system in SCVs, it is tempting to speculate that these variants are not only locked in a colonization state, but also in a biofilm-forming state.

Overall, the *S. aureus* SigB factor was described as having a role in stress resistance [8, 54], a role in biofilm formation [13-15] and a positive influence for the expression of adhesins needed for host colonization [4, 5, 7]. Despite these clear links between SigB and bacterial factors influencing pathogenesis, the role of SigB *in vivo* is still not well established. As examples, no influence of SigB has been detected in experimental endocartitis [5, 55, 56] or in an abscess model [43, 54] while a role for SigB was demonstrated in catheter-associated infections [57] and in a murine model of bacteria-induced septic arthritis [58]. The results of the present study suggest that SigB does not significantly contribute to the short-term colonization of the mouse mammary glands (Fig. 5A). This however remains to be confirmed in a model in which a suckling pressure can be added (or milking by analogy to the cow). Indeed, we have previously demonstrated that differences in the colonization of the mammary gland between $FnBP^+$ and $FnBP^-$ strains in the mouse mastitis model could only be observed under suckling pressure where adhesion is thought to be critical [59]. On the other hand, our results may certainly suggest a long-term influence of SigB in the pathogenesis of *S. aureus*, for instance, in the establishment of a persistence or chronic disease. Indeed, the significant difference we observed in the number of SCVs recovered from mice infected with Newbould and NewbouldΔ*sigB* following gentamicin treatment supports this hypothesis since SCVs have been associated with chronic infections.

Our results suggest that SigB is involved in the emergence of the SCV phenotype resulting from an exposure to a sub-inhibitory concentration of aminoglycoside antibiotics. To our knowledge, this is the first study supporting the idea that a regulatory component may influence the emergence of SCVs in *S. aureus*, or taken more generally, in a Gram positive bacteria. However, our results only suggest that SigB is required to generate quantitatively more SCVs in a population of *S. aureus* exposed to aminoglycosides and questions about the specific role SigB in the formation and/or the survival of SCVs remains to be fully answered. Furthermore, it should be kept in mind that the SCV phenotype is likely to be heterogeneous in regard to the type of auxotrophies and antibiotic resistance patterns that may occur or even

to the relative ability of SCVs to revert to the wild-type phenotype, and that this heterogeneity could reflect the existence of various mechanisms leading to the occurrence of SCVs [39]. The mechanism(s) of SCV formation may fundamentally imply genetic events such as random mutations, genetic mechanisms such as that involved in phase variation of pathogenic bacteria and/or regulatory mechanisms [27, 38, 39]. Moreover, genetic and regulatory mechanisms are not necessarily mutually exclusive. For example, it has been shown that SigB may regulate a genetic mechanism involved in biofilm phenotypic variation in Staphylococci [60]. While the specific mechanism(s) of SCV formation still remains largely unresolved, our results suggest that regulatory components may be required for the emergence of this phenotype upon exposure to aminoglycosides.

The association found here between the stress-associated sigma factor B of *S. aureus* and the SCV phenotype raises the possibility that this phenotype confers a selective advantage to the bacterium in difficult environments. In this regard, the slower metabolism of SCVs may decrease their susceptibility to antibiotics and other harmful molecules, thus enabling the survival of the bacterium in stringent conditions and the establishment of chronic and difficult-to-treat infections. Furthermore, we have previously shown that SCVs isolated from CF patients also express stress-related genes such as *clp, htrA, katA, spoVG* and *proP* at a higher level than prototypical strains [7], which is supporting the hypothesis that SCVs may effectively be better equipped to survive in harsh environments. The increased ability of these variants to form biofilm is likely to also play a role in the survival of the bacterium since biofilms are thought to confer protection from the immune host response and action of antibiotics [17]. This study supports the hypothesis that *S. aureus* virulence regulators, such as SigB, may influence the emergence of the SCV phenotype and the formation of biofilms which are frequently associated to chronic infections. Consequently, it may be possible to prevent or, at least, minimize the establishment of these persistent infections by altering the activity of specific *S. aureus* regulatory networks.

4. MATERIALS AND METHODS

4.1. Bacterial strains and growth conditions

S. aureus Newbould (ATCC 29740) was used as a representative for the wild-type prototypical phenotype and because it was originally isolated from a case of bovine mastitis and is thus more relevant for the mouse mastitis infection model used in this study (see below). We already validated the choice of Newbould as a control/prototypical strain [7]. Newbould indeed behaves like other prototypical strains according to growth rate and expression of virulence factors. NewbouldΔ*sigB*, in which *sigB* had been disrupted by the *emrA* cassette, was used to evaluate the importance of *sigB* in a prototypical background. CF03 and CF07 are previously characterized menadione-auxotroph SCVs isolated from CF patients [7]. CF07Δ*sigB* has been constructed by disrupting the *sigB* gene in CF07 by the insertion of the *ermA* cassette by homologous recombination [6]. Strains 3231 and SCV Heba3231 are derivatives of the same genetic background that have been isolated from the same milk sample of a case of chronic bovine mastitis [32].

For all experiments presented in this study, 18-20 h broth cultures were used to inoculate brain heart infusion (BHI) broth (BD, ON, Canada) at an A_{595nm} of 0.1. The bacteria were then grown aerobically at 35°C with shaking (225 rpm) until reaching a specific growth phase. The A_{595nm} values for the early exponential growth phase were, respectively, 0.4 and 0.25 for the prototypical and the SCV strains. In the case of SCVs, cultures were carried out for at least 12h after the last sampling point in order to insure that no reversion to the non-SCV phenotype (increased growth rate and formation of large prototypical colonies) had occurred during the experiment. To study the effect of sub-inhibitory concentrations of gentamicin and tobramycin on the expression of target genes, bacteria were collected when the culture reached an A_{595nm} values between 0.9 and 1.0 (exponential phase of growth). The growth of CF07 was

prototypical when its growth medium contained 1 μg ml^{-1} of supplemental menadione (Sigma-Aldrich, ON, Canada).

4.2. Growth curves

Samples were taken at different time points for the determination of CFUs by plating 10-fold dilutions on trypticase soy agar (TSA) medium (BD, ON, Canada). Plates were incubated at 35°C for 24 and 48 h for prototypical and SCV strains, respectively.

4.3. Antibiotic susceptibility

The minimal inhibitory concentrations (MICs) of tobramycin and gentamicin for all strains were determined by a broth microdilution technique, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines [61]. A sub-inhibitory concentration (0.5 × MIC) of each antibiotic for each strain was used for the quantification of SCVs formed under gentamicin and tobramycin exposure.

4.4. Quantification of small-colony variants

Broth cultures (18-20 h) were used to inoculate BHI broth at a dilution of 1:100, supplemented or not with 0.5 × the MIC for gentamicin or tobramycin. Cultures were incubated 18 h at 35°C with shaking (225 rpm) and then adjusted to an A_{595nm} of 2.0 in PBS at 4°C. Determination of CFU and SCV colonies was done by serial dilution plating. SCV counts were obtained by plating on TSA containing gentamicin at a concentration of 4 μg ml^{-1} followed by an incubation of 48 h at 35°C. The frequency of SCV formation was determined as the number of SCVs per total CFU counts on antibiotic free TSA. The pinpoint colonies selected by this method were confirmed to be SCVs by streaking several of them on TSA plates. Non-SCV colonies (larger) were also rarely selected on TSA containing gentamicin, but were not considered in our analysis.

4.5. Quantitative PCR (qPCR)

Bacteria were collected at specific time points and treated with RNAprotect (QIAGEN). RNA was extracted from the pellets after bacterial lysis with 200 μg ml^{-1} lysostaphin (Sigma-Aldrich) during 1 h at room temperature using the RNeasy Mini kit and the RNase-free DNase set (QIAGEN). A second DNase treatment was also done with the DNA-free kit (Applied Biosystems/Ambion, CA, USA). One μg of total RNA was reverse transcribed with 0.5 mM deoxynucleotide phosphate, 50 ng of random hexamers and 200 U of Invitrogen Superscript II reverse transcriptase, according to the manufacturer's recommendations (Invitrogen, ON, Canada). RNA was hydrolyzed and the cDNAs were purified with the QIAquick PCR purification kit (QIAGEN). One microliter of the cDNA preparation was amplified on the Stratagene MX3000P Real-Time PCR instrument with the Jump Start Taq DNA polymerase (Sigma-Aldrich), SYBR Green and 100 nM of the following primers:

asp23-RT-FWD 5'-TCGCTGCACGTGAAGTTAAA-3',
asp23-RT-REV 5'-CAGCAGCTTGTTTTTCACCA-3',
fnbA268-RT-FWD 5'-ACAAGTTGAAGTGGCACAGCC-3',
fnbA341-RT-REV 5'-CCGCTACATCTGCTGATCTTGTC-3',
hld-RT-FWD 5'-TAATTAAGGAAGGAGTGATTTCAATG-3'
hld-RT-REV 5'-TTTTTAGTGAATTTGTTCACTGTGTC-3'
hla-RT-FWD 5'-AATGAATCCTGTCGCTAATGCCGC-3'
hla-RT-REV 5'-CTGAAGGCCAGGCTAAACCACTTT-3'
sarA-RT-FWD 5'-CAAACAACCACAAGTTGTTAAAGC-3'
sarA-RT-REV 5'-TGTTTGCTTCAGTGATTCGTTT-3'
16SrRNA-RT-FWD 5'- TCGTTTAACACGTTTAGGTTCA-3',
16SrRNA-RT-REV 5'- GAACTGTATCAGTTGGTTTCGCAC-3',
gyrB-RT-FWD 5'-GGTGCTGGGCAAATACAAGT-3',
gyrB-RT-REV 5'-TCCCACACTAAATGGTGCAA-3'.

Reaction mixtures were denatured for 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 60°C and 1 min 30 s at 72°C. Dissociation and standard curves were obtained to insure the specificity and the efficiency of reactions. cDNA synthesis reactions without reverse transcriptase were also routinely carried out. The relative transcription ratios were calculated by using the cycle threshold (C_t) of the 16S RNA or *gyrB* of each condition as the calibrator (*n*-fold expression = $2^{-\Delta Ct}$, where ΔC_t represents the difference between the C_t of the gene studied and the C_t of the 16S RNA or *gyrB* for each condition).

4.6. Biofilm formation

Biofilm formation was evaluated by spectrophotometry in microplates using crystal violet staining, as previously described with few modifications [62]. Briefly, strains were cultured from frozen stocks onto blood agar plates and incubated at 35°C overnight. Three colonies were then inoculated into 7 ml of BHI containing 0.25% of supplemental glucose and incubated at 35°C for 18 h with shaking at 225 rpm. This culture was then diluted to 0.5 McFarland in BHI 0.25% glucose and transferred into wells of a flat-bottom polystyrene microtiter plate containing half volume of the same medium. To test the effect of gentamicin on biofilm formation by Newbould and NewbouldΔ*sigB*, the antibiotic was added in each well at a final concentration of 0.5 × MIC. The plates were then incubated at 35°C for 24 or 48 h. The supernatant was then discarded and the wells were delicately washed three times with 200 µl of PBS. The plates were dried, stained for 30 min with crystal violet, washed twice with 200 µl of water and allowed to dry again. A volume of 200 µl of 95% ethanol was added to each well and plates were incubated at room temperature for 1 h with frequent agitation. The absorbance of each well was then measured at 560 nm using a plate reader (Bio-Tek Instruments). The results were collected from at least three independent experiments in which the biofilm formation of each culture tested was evaluated in four replicates.

4.7. Quantification of SCVs in the mouse mastitis model

The mouse mastitis model of *S. aureus* infection has been previously described [33, 59, 62, 63]. Briefly, 1 h following removal of the 12-14 day-old offspring, lactating CD-1 mice (Charles River, St.-Constant, Québec, Canada) were anesthetized with ketamine and xylazine at 87 and 13 mg kg^{-1} of body weight, respectively, and mammary glands were inoculated under a binocular. Mammary ducts were exposed by a small cut at the near end of the teats and a 100 μl-bacterial suspension, containing approximately 100 CFU in phosphate-buffered saline (PBS), was injected through the teat canal using a 32-gauge blunt needle. Two glands (fourth on the right [R4] and fourth on the left [L4], from head to tail) were inoculated for each animal with Newbould and NewbouldΔ*sigB*, respectively. Mice were injected intravenously with 0.5 mg kg^{-1} of body weight of gentamicin at 2 h and 10 h post-infection. Mammary glands were aseptically harvested after 18 h of infection and the bacterial content was evaluated after tissue homogenization in PBS by serial logarithmic dilutions and plating on TSA for CFU counting. TSA containing 1.5 μg ml^{-1} of gentamicin were used in order to determine the counts of SCVs present in the glands. The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke (QC, Canada) approved these experiments and the guidelines of the Canadian Council on Animal Care were respected during all the procedures.

4.8. Statistical analysis

One-way analysis of variance followed by Dunnett's multiple comparisons test or Tukey's multiple comparisons test were used when several conditions or strains were compared at the same time whereas unpaired *t*-tests were used when only two conditions were compared. Statistical analyses of qPCR data were done on mean ΔC_t. For the *in vivo* SCV formation experiments, statistical significance was evaluated by the non-parametric Kruskal-Wallis one-way analysis of variance followed by the Dunn's multiple comparisons test. CFU counts or SCV frequencies were transformed in based-10 logarithm values before being used for

statistical analyses that were carried out with the GraphPad Prism Software (v.5.00). Statistical tests used for the analysis of each experiment are specified in figure legends.

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REFERENCES

[1] Archer GL. *Staphylococcus aureus*: a well-armed pathogen. Clin Infect Dis 1998;26(5):1179-81.

[2] Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol 2003;48(6):1429-49.

(Wright *et al.*) Novick RP, Geisinger E. Quorum sensing in staphylococci. Annu Rev Genet 2008;42:541-64.

[4] Bischoff M, Dunman P, Kormanec J, Macapagal D, Murphy E, Mounts W, *et al.* Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. J Bacteriol 2004;186(13):4085-99.

[5] Entenza JM, Moreillon P, Senn MM, Kormanec J, Dunman PM, Berger-Bachi B, *et al.* Role of σ^B in the expression of *Staphylococcus aureus* cell wall adhesins ClfA and FnbA and contribution to infectivity in a rat model of experimental endocarditis. Infect Immun 2005;73(2):990-8.

[6] Mitchell G, Lamontagne CA, Brouillette E, Grondin G, Talbot BG, Grandbois M,

et al. *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. Mol Microbiol 2008;70(6):1540-55.

[7] Moisan H, Brouillette E, Jacob CL, Langlois-Begin P, Michaud S, Malouin F. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J Bacteriol 2006;188(1):64-76.

[8] Price CW. General stress response. In: Sonenshein AL, ed. *Bacillus subtilis* and its closest relatives; from genes to cells. Washington, D.C.: ASM; 2002:369-84.

[9] Deora R, Tseng T, Misra TK. Alternative transcription factor σ^{SB} of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *sar*. J Bacteriol 1997;179(20):6355-9.

[10] Cheung AL, Zhang G. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. Front Biosci 2002;7:d1825-42.

[11] Bischoff M, Entenza JM, Giachino P. Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. J Bacteriol 2001;183(17):5171-9.

[12] Boles BR, Horswill AR. *agr*-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog 2008;4(4):e1000052.

[13] Lauderdale KJ, Boles BR, Cheung AL, Horswill AR. Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infect Immun 2009;77(4):1623-35.

[14] Kim JH, Kim CH, Hacker J, Ziebuhr W, Lee BK, Cho SH. Molecular characterization of regulatory genes associated with biofilm variation in a *Staphylococcus aureus* strain. J Microbiol Biotechnol 2008;18(1):28-34.

[15] Rachid S, Ohlsen K, Wallner U, Hacker J, Hecker M, Ziebuhr W. Alternative transcription factor σ^B is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. J Bacteriol 2000;182(23):6824-6.

[16] Beenken KE, Blevins JS, Smeltzer MS. Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. Infect Immun 2003;71(7):4206-11.

[17] Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol 2002;292(2):107-13.

[18] Archibald LK, Gaynes RP. Hospital-acquired infections in the United States. The importance of interhospital comparisons. Infect Dis Clin North Am 1997;11(2):245-55.

[19] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284(5418):1318-22.

[20] Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2003;2(2):114-22.

[21] Gruet P, Maincent P, Berthelot X, Kaltsatos V. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv Drug Deliv Rev 2001;50(3):245-59.

[22] Sears PM, McCarthy KK. Management and treatment of staphylococcal mastitis. Vet Clin North Am Food Anim Pract 2003;19(1):171-85, vii.

[23] Sutra L, Poutrel B. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. J Med Microbiol 1994;40(2):79-89.

[24] Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzon M, Peris C, *et al.* Role of biofilm-associated protein *bap* in the pathogenesis of bovine *Staphylococcus aureus*. Infect Immun 2004;72(4):2177-85.

[25] Vasudevan P, Nair MK, Annamalai T, Venkitanarayanan KS. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol 2003;92(1-2):179-85.

[26] Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 2002;15(2):194-222.

[27] Proctor RA, von Eiff C, Kahl BC, *et al.* Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 2006;4(4):295-305.

[28] von Eiff C, Proctor RA, Peters G. *Staphylococcus aureus* small colony variants: formation and clinical impact. Int J Clin Pract Suppl 2000(115):44-9.

[29] Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, *et al.* Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. J Infect Dis 1998;177(4):1023-9.

[30] Sompolinsky D, Cohen M, Ziv G. Epidemiological and biochemical studies on thiamine-less dwarf-colony variants of *Staphylococcus aureus* as etiological agents of bovine mastitis. Infect Immun 1974;9(2):217-28.

[31] Sadowska B, Bonar A, von Eiff C, Proctor RA, Chmiela M, Rudnicka W, *et al.* Characteristics of *Staphylococcus aureus*, isolated from airways of cystic fibrosis patients, and their small colony variants. FEMS Immunol Med Microbiol 2002;32(3):191-7.

[32] Atalla H, Gyles C, Jacob CL, Moisan H, Malouin F, Mallard B. Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. Foodborne Pathog Dis 2008;5(6):785-99.

[33] Brouillette E, Martinez A, Boyll BJ, Allen NE, Malouin F. Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*. FEMS Immunol Med Microbiol 2004;41(1):35-41.

[34] Alexander EH, Hudson MC. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. Appl Microbiol Biotechnol 2001;56(3-4):361-6.

[35] Singh R, Ray P, Das A, Sharma M. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. J Med Microbiol 2009;58(Pt 8):1067-73.

[36] Balwit JM, van Langevelde P, Vann JM, Proctor RA. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J Infect Dis 1994;170(4):1033-7.

[37] Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, *et al.* Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 2006;103(52):19890-5.

[38] Massey RC, Buckling A, Peacock SJ. Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. Curr Biol 2001;11(22):1810-4.

[39] Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. Int J Med Microbiol 2003;293(6):427-35.

[40] von Eiff C, Bettin D, Proctor RA, Rolauffs B, Lindner N, Winkelmann W, *et al.* Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. Clin Infect Dis 1997;25(5):1250-1.

[41] Lehtolainen T, Shwimmer A, Shpigel NY, Honkanen-Buzalski T, Pyorala S. *In vitro* antimicrobial susceptibility of *Escherichia coli* isolates from clinical bovine mastitis in Finland and Israel. J Dairy Sci 2003;86(12):3927-32.

[42] MacLeod DL, Prescott JF. The use of liposomally-entrapped gentamicin in the treatment of bovine *Staphylococcus aureus* mastitis. Can J Vet Res 1988;52(4):445-50.

[43] Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ. σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. J Bacteriol 2002;184(19):5457-67.

[44] Cheung A. Global regulation of virulence determinants in *Staphylococcus aureus*. In: Honeyman AL, Friedman H, Bendinelli M editors. *Staphylococcus aureus* Infection and Disease, New York: Kluwer academic/Plenum Publishers; 2001:295-322.

[45] Senn MM, Bischoff M, von Eiff C, Berger-Bachi B. σ^B activity in a *Staphylococcus aureus hemB* mutant. J Bacteriol 2005;187(21):7397-406.

[46] Kahl BC, Belling G, Becker P, Chatterjee I, Wardecki K, Hilgert K, *et al.* Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infect Immun 2005;73(7):4119-26.

[47] Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, Proctor RA, *et al.* A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*. J Bacteriol 2008;190(19):6351-64.

[48] Newbould FH. Antibiotic treatment of experimental *Staphylococcus aureus* infections of the bovine mammary gland. Can J Comp Med 1974;38(4):411-6.

[49] Musher DM, Baughn RE, Templeton GB, Minuth JN. Emergence of variant forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. J Infect Dis 1977;136(3):360-9.

[50] Doss SA, Tillotson GS, Amyes SG. Effect of sub-inhibitory concentrations of antibiotics on the virulence of *Staphylococcus aureus*. J Appl Bacteriol 1993;75(2):123-8.

[51] Lin JT, Connelly MB, Amolo C, Otani S, Yaver DS. Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. Antimicrob Agents Chemother 2005;49(5):1915-26.

[52] O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, *et al.* A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol 2008;190(11):3835-50.

[53] O'Gara JP. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol Lett 2007;270(2):179-88.

[54] Chan PF, Foster SJ, Ingham E, Clements MO. The *Staphylococcus aureus* alternative sigma factor σ^B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J Bacteriol 1998;180(23):6082-9.

[55] Kupferwasser LI, Yeaman MR, Nast CC, Kupferwasser D, Xiong YQ, Palma M, *et al.* Salicylic acid attenuates virulence in endovascular infections by targeting global regulatory pathways in *Staphylococcus aureus*. J Clin Invest 2003;112(2):222-33.

[56] Xiong YQ, Willard J, Yeaman MR, Cheung AL, Bayer AS. Regulation of *Staphylococcus aureus* alpha-toxin gene (*hla*) expression by *agr*, *sarA*, and *sae in vitro* and in experimental infective endocarditis. J Infect Dis 2006;194(9):1267-75.

[57] Lorenz U, Huttinger C, Schafer T, Ziebuhr W, Thiede A, Hacker J, *et al.* The alternative sigma factor σ^B of *Staphylococcus aureus* modulates virulence in experimental central venous catheter-related infections. Microbes Infect 2008;10(3):217-23.

[58] Jonsson IM, Arvidson S, Foster S, Tarkowski A. Sigma factor B and RsbU are required for virulence in *Staphylococcus aureus*-induced arthritis and sepsis. Infect Immun 2004;72(10):6106-11.

[59] Brouillette E, Talbot BG, Malouin F. The fibronectin-binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis. Infect Immun 2003;71(4):2292-5.
[60] Valle J, Vergara-Irigaray M, Merino N, Penades JR, Lasa I. σ^B regulates IS256-mediated *Staphylococcus aureus* biofilm phenotypic variation. J Bacteriol 2007;189(7):2886-96.
[61] Clinical and Laboratory Standards Institue (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved Standard. 2006.
[62] Brouillette E, Hyodo M, Hayakawa Y, Karaolis DK, Malouin F. 3',5'-cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. Antimicrob Agents Chemother 2005;49(8):3109-13.
[63] Brouillette E, Malouin F. The pathogenesis and control of *Staphylococcus aureus*-induced mastitis: study models in the mouse. Microbes Infect 2005;7(3):560-8.

CHAPITRE 3

Staphylococcus aureus sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-*N*-oxide

3.1. Introduction de l'article

Le but de cette étude était d'investiguer l'association entre l'activité de SigB, l'émergence de SCVs et la production de biofilm chez *S. aureus* lorsqu'exposé aux exoproduits de *P. aeruginosa*. L'étudiant David Lalonde Séguin et moi avons effectué les expériences de microbiologie et supervisé la stagiaire Ann-Élise Asselin. J'ai effectué les expériences de PCR quantitatives en temps réel. Le Pr Éric Déziel nous a fourni des souches mutantes de *P. aeruginosa*. Le Dr André M. Cantin, le Pr Éric H. Frost et la Dre Sophie Michaud ont contribué intellectuellement au projet. J'ai rédigé l'ébauche de l'article, effectué la conception des figures et participé à la correction du manuscrit jusqu'à sa publication.

Référence de l'article :

Mitchell, G., Séguin, D.L., Asselin, A.E., Déziel, E., Cantin, A.M., Frost, E.H., Michaud, S., and Malouin, F. (2010). *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. BMC Microbiol. *30*, 10:33.

3.2. Résumé en français de l'article

CONTEXTE : *Staphylococcus aureus* et *Pseudomonas aeruginosa* sont souvent retrouvés simultanément dans les voies respiratoires des patients FK. Il a été montré précédemment que l'exoproduit 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) de *P. aeruginosa* inhibe la croissance de *S. aureus* et provoque l'émergence de SCVs. La présence de SCVs de *S. aureus* ainsi que la formation de biofilm ont été associées aux infections chroniques chez les patients FK. RÉSULTATS : Nous avons démontré que le HQNO stimule *S. aureus* à former un biofilm en association avec la formation de SCVs. L'émergence de SCVs et la production de biofilm suite à l'exposition au HQNO étaient dépendantes de l'activité du facteur sigma alternatif sigma B (SigB), lié à la réponse aux stress et à l'état de colonisation. L'analyse de l'expression de gènes a révélé que l'exposition d'une souche prototype au HQNO active SigB, menant à une augmentation de l'expression d'un gène encodant une protéine liant la fibronectine (FnBPA) et du gène *sarA* associé à la formation de biofilms. Inversement, le régulateur de gènes accessoires dépendant du *quorum-sensing* (*agr*) et le gène encodant Hla étaient réprimés par le HQNO. Des expériences utilisant du surnageant de culture obtenu de la souche PAO1 de *P. aeruginosa* et un modèle de coculture utilisant des chambres doubles ont confirmé que *P. aeruginosa* stimule la formation de biofilm et active SigB chez une souche de *S. aureus* isolée d'un patient FK. De plus, le surnageant de mutants de *P. aeruginosa* incapables de produire du HQNO induisait la formation de biofilm par *S. aureus* à une moindre mesure que la souche sauvage seulement lorsque SigB était fonctionnel. CONCLUSIONS : Ces résultats suggèrent que *S. aureus* répond au HQNO de *P. aeruginosa* en formant des SCVs et du biofilm suite à l'activation de SigB. Ce phénomène pourrait contribuer à l'établissement d'infections chroniques chez les patients FK.

3.3. L'article scientifique

Staphylococcus aureus sigma B-dependent emergence of small-colony variants and biofilm production following exposure to _Pseudomonas aeruginosa_ 4-hydroxy-2-heptylquinoline-_N_-oxide

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ABSTRACT

Background: *Staphylococcus aureus* and *Pseudomonas aeruginosa* are often found together in the airways of cystic fibrosis (CF) patients. It was previously shown that the *P. aeruginosa* exoproduct 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) suppresses the growth of *S. aureus* and provokes the emergence of small-colony variants (SCVs). The presence of *S. aureus* SCVs as well as biofilms have both been associated with chronic infections in CF. **Results:** We demonstrated that HQNO stimulates *S. aureus* to form a biofilm in association with the formation of SCVs. The emergence of SCVs and biofilm production under HQNO exposure was shown to be dependent on the activity of the stress- and colonization-related alternative sigma factor B (SigB). Analysis of gene expression revealed that exposure of a prototypical *S. aureus* strain to HQNO activates SigB, which was leading to an increase in the expression of the fibronectin-binding protein A and the biofilm-associated *sarA* genes. Conversely, the quorum sensing accessory gene regulator (*agr*) system and the α-hemolysin gene were repressed by HQNO. Experiments using culture supernatants from *P. aeruginosa* PAO1 and a double chamber co-culture model confirmed that *P. aeruginosa* stimulates biofilm formation and activates SigB in a *S. aureus* strain isolated from a CF patient. Furthermore, the supernatant from *P. aeruginosa* mutants unable to produce HQNO induced the production of biofilms by *S. aureus* to a lesser extent than the wild-type strain only in a *S. aureus* SigB-functional background. **Conclusions:** These results suggest that *S. aureus* responds to HQNO from *P. aeruginosa* by forming SCVs and biofilms through SigB activation, a phenomenon that may contribute to the establishment of chronic infections in CF patients.

BACKGROUND

Although cystic fibrosis (CF) is fundamentally a genetic disorder, the majority of patients with CF may ultimately succumb to respiratory failure subsequent to chronic bacterial infections [1]. In early childhood, lungs of CF patients are often infected with *Staphylococcus aureus* and *Haemophilus influenzae*, but these organisms are usually outnumbered by *Pseudomonas aeruginosa* as patients become older. However, *S. aureus* often persists in the airways of CF patients and the role of *S. aureus* in the progression of CF patients to respiratory failure is not yet understood whereas infections with *P. aeruginosa* is considered as one of the main factors for a decline in lung function and mortality [1]. Interestingly, both organisms are commonly co-isolated from CF airways [2, 3].

Infections with mixed microbial communities are common, although very little is known about the importance and the impact of interspecies interactions [4]. It is now becoming obvious that the different bacteria found in CF airways interact together in several different ways [5-10]. One possibility is that polymicrobial interactions influence pathogenic processes such as biofilm formation [1, 9]. Accordingly, the biofilm lifestyle is now recognized as an integrated and complex polymicrobial community and it is thought that cell-to-cell interspecies signals play a role in the control of this behavior [11].

It has recently been shown that prolonged growth of *S. aureus* with physiological concentrations of the *P. aeruginosa* exoproduct 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) selects for a sub-population of slow-growing respiratory deficient *S. aureus* named small-colony variants (SCVs) [2]. The respiratory deficiency of SCVs provides resistance to aminoglycoside antibiotics, which can contribute to microbial persistence during antibiotherapy [12]. Furthermore, it has been recently demonstrated that SCV selection is a

survival strategy of *S. aureus* against *P. aeruginosa* [13]. *S. aureus* SCVs are often isolated from chronic infections [12], as in the case of lung infections of CF patients [14-16]. Several studies have shown that *S. aureus* SCVs possess an increased capacity to invade and persist in host cells [14, 15, 17], which is thought to confer the bacterium protection against the immune system and the action of antibiotics [17, 18]. Using SCVs isolated from cystic fibrosis patients, we have previously demonstrated that the alternative transcription sigma factor B (SigB) influences the expression of several virulence factors and is associated with an increased ability to adhere, invade and persist within host cells [15, 19]. Furthermore, our more recent results suggest that SigB is involved in the emergence of SCVs under aminoglycoside pressure [20], which suggests that the appearance of SCVs may be a regulated process influenced by environmental cues. Our current hypothesis is that SigB plays an important role in the establishment of chronic and difficult-to-treat *S. aureus* infections.

SigB is involved in the response to environmental stresses such as during stationary phase, heat exposure and change in osmotic pressure [21]. Moreover, the activity of SigB positively influences the expression of several cell-surface proteins whereas it down-regulates a variety of toxins [22], which suggest an important role for SigB in pathogenesis. The effect of SigB on virulence gene expression can be direct or indirect, since the genes regulated by SigB also include at least another global regulator of virulence, *sarA* (Staphylococcal accessory regulator) [22, 23]. SarA modulates the expression of several virulence factors either by stimulating RNAIII transcription or by pathway(s) independent of the *agr* (accessory gene regulator) system [24]. In turn, it is proposed that the quorum-sensing *agr* system controls the transition from colonization to dissemination by up-regulating the expression of several exotoxins and proteolytic enzymes and by repressing the expression of cell-surface proteins involved in colonization [25]. *agr* [26], SigB [27, 28] and SarA [29] are known to influence the formation of biofilms by *S. aureus*.

At least two different mechanisms of biofilm formation exist in *S. aureus* [26, 29-33]. The first mechanism implies the production of the polysaccharide intercellular adhesin (PIA), which requires the *ica* gene cluster, whereas the second mechanism is *ica*-independent. With opposite effects, SarA and *agr* are both involved in the *ica*-independent mechanism of biofilm formation. SarA is thought to be indirectly required for the initial attachment step to biological matrices [29, 32, 33], while *agr* is controlling the dispersal process of biofilms [26]. Recently, Lauderdale *et al.* [30] have shown that SigB is an essential regulator of the *ica*-independent biofilm formation and suggested that SigB acts upstream of the *agr* system, allowing the formation of biofilm to be regulated as a function of environmental factors. Noteworthy, biofilms have been linked to chronic infections, especially in the case of those found in the airways of CF patients [1, 34], and an increased formation of biofilms has been associated with the SCV phenotype [20, 35].

The aim of this study was to investigate the association between the activity of SigB, the emergence of SCVs and biofilm production in *S. aureus* when exposed to *P. aeruginosa* HQNO.

RESULTS

HQNO inhibits the growth of normal strains and provokes the emergence of SCVs in *S. aureus*. Fig. 1 confirms that HQNO suppresses the growth of *S. aureus* and causes the emergence of SCVs. Isolates CF1A-L and CF1D-S are two related strains co-isolated from a CF patient which have a normal and a SCV phenotype, respectively (see Methods). At a concentration of 10 μg/ml, HQNO significantly attenuated the growth of CF1A-L ($P < 0.01$ from 6 to 12 h of growth; two-way ANOVA followed by a Bonferroni's post test) whereas HQNO had no apparent effect on the growth of CF1D-S which was already significantly

slower than that of CF1A-L in the absence of HQNO ($P < 0.001$ from 6 to 12 h of growth; two-way ANOVA followed by a Bonferroni's post test) (Fig. 1A). Similar observations were also reproduced with other strains (two normal and one SCV; data not shown). Fig. 1B shows that an overnight treatment with HQNO provokes the emergence of SCVs from CF1A-L, as determined by plating the culture on solid medium containing a concentration of gentamicin selective for the SCV phenotype. Very little or no SCV were detected on gentamicin plates when cultures were not exposed to HQNO (Fig. 1B). Hence, this technique allowed detection and quantification of SCVs emerging during the growth of normal bacteria exposed or not to HQNO. This approach was thus used to distinguish the transitory suppression of growth of normal *S. aureus* by HQNO from the emerging slow-growing SCVs for which gentamicin resistance and slow growth persist even after removal of HQNO. Fig. 1C shows that 10 µg of HQNO/ml significantly increased the presence of SCVs in cultures of the prototypical strains ATCC 29213, Newman and Newbould as well as of the other normal strains isolated from CF patients CF03-L, CF07-L and CF1A-L. Differences in HQNO-mediated SCV emergence between strains were not significant, except between ATCC 29213 and Newbould ($P < 0.01$; one-way ANOVA followed by a Tuckey's post test). These results corroborate that HQNO generally suppresses the growth of normal *S. aureus* populations and provokes the emergence of SCVs from strains of different origins.

HQNO stimulates biofilm production in normal strains but does not alter high biofilm production in SCVs. Several pairs of related normal and SCVs strains were used in order to study the effect of HQNO on biofilm production by *S. aureus*. Fig. 2A shows that SCVs produce significantly more biofilm than their normal counterparts. The use of the strain Newbould*hemB* (which is a stable laboratory-derived SCV) ensured that SCVs (and not revertants) are indeed responsible for this increase in biofilm production (at least in the case of Newbould*hemB*). Furthermore, as shown in Mitchell *et al.* [20], supplementation of the SCV strains CF03 and CF07 with menadione abolished this phenomenon and thus demonstrated

that if there was a reversion of SCVs to the normal phenotype, the biofilm production would be greatly reduced.

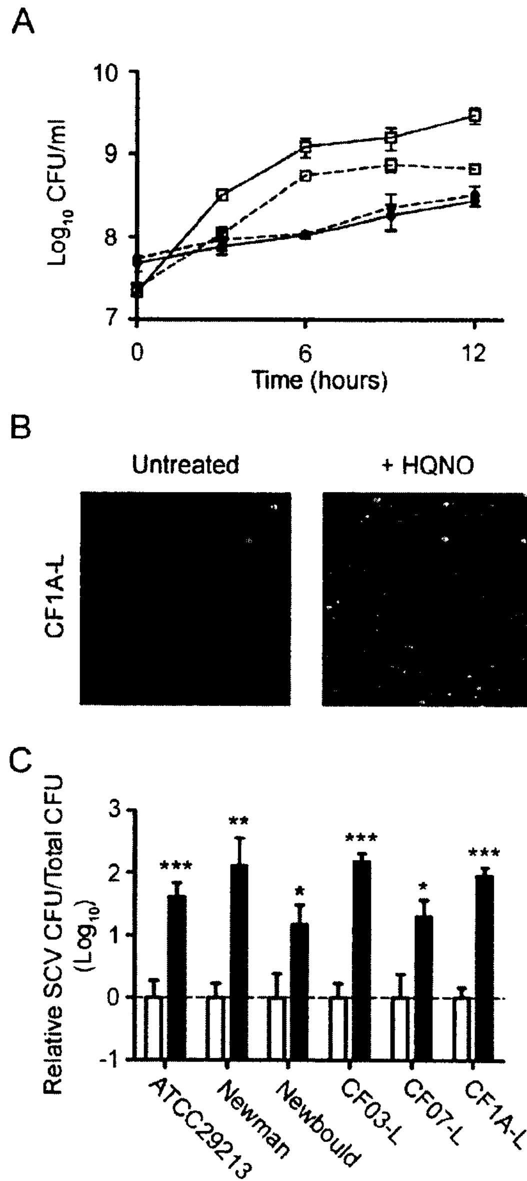


FIGURE 1. HQNO inhibits the growth of normal *S. aureus* strains and provokes the emergence of SCVs. (A) Growth curves of the normal strain CF1A-L (□) and the SCV CF1D-S (●) exposed (dotted lines) or not (solid lines) to 10 µg/ml of HQNO. (B) Pictures show SCV colonies grown on agar containing a selective concentration of gentamicin following or not an overnight treatment of strain CF1A-L with 10 µg/ml of HQNO. (C) Relative number of SCV CFUs recovered after 18 h of growth from strains ATCC 29213, Newman, Newbould, CF03-L, CF07-L and CF1A-L following (black bars) or not (open bars) treatments with 10 µg/ml of HQNO. Data are presented as means with standard deviations from at least three independent experiments. Results are normalized to the non exposed condition for each strain (dotted line). Significant differences between untreated and HQNO-treated conditions are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired *t*-test).

Besides, the presence of HQNO at 10 µg/ml did stimulate biofilm production in the normal strains (Fig. 2B-C). This observation was statistically significant for the normal strains ATCC 29213, Newman, Newbould, CF03-L, CF07-L and CF1A-L whereas HQNO had no detectable effect on the already high biofilm production of the SCV strains Newbould*hemB*, CF03-S, CF07-S and CF1D-S (Fig. 2C). Moreover, CF03-L produced significantly more biofilm than ATCC 29213 and Newman in presence of HQNO, revealing that the amplitude of the response of normal strains to HQNO may individually differs (Fig. 2C). Interestingly, an overnight exposure to 10 µg/ml of HQNO resulted in a significant increase in biofilm production ($P < 0.05$) for strain Newman, CF03-L and CF1A-L even after sub-culturing strains in HQNO-free medium (data not shown). This indicates that an exposure of *S. aureus* to HQNO may result in a sustained increase in biofilm production. Overall, these results suggest that HQNO increases biofilm production in normal *S. aureus* strains and that the sustained effect of HQNO on biofilm production in subsequent HQNO-free medium may result from the relative increase in the sub-population of SCVs which are good biofilm producers.

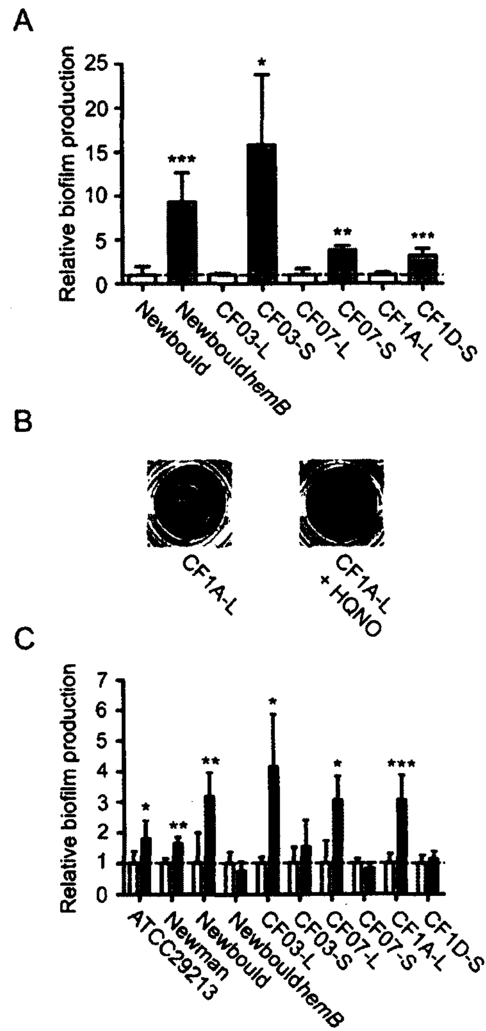


FIGURE 2. HQNO stimulates biofilm production in normal strains but does not alter high biofilm production in SCVs. (A) Relative biofilm production in related normal (open bars) and SCV (grey bars) strains. Results are normalized to the normal strain for each pair (dotted line). (B) Pictures show the biofilm formation of the normal strain CF1A-L in the absence or in the presence of HQNO as detected by crystal violet staining. (C) Relative biofilm production in strains exposed (black bars) or not (open bars) to 10 µg/ml of HQNO. Results are normalized to the unexposed condition for each strain (dotted line). Data are presented as means with standard deviations from at least three independent experiments. Significant differences between normal and SCV strains (-L and -S suffixes, respectively) or between unexposed and

HQNO-exposed conditions are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired t-test).

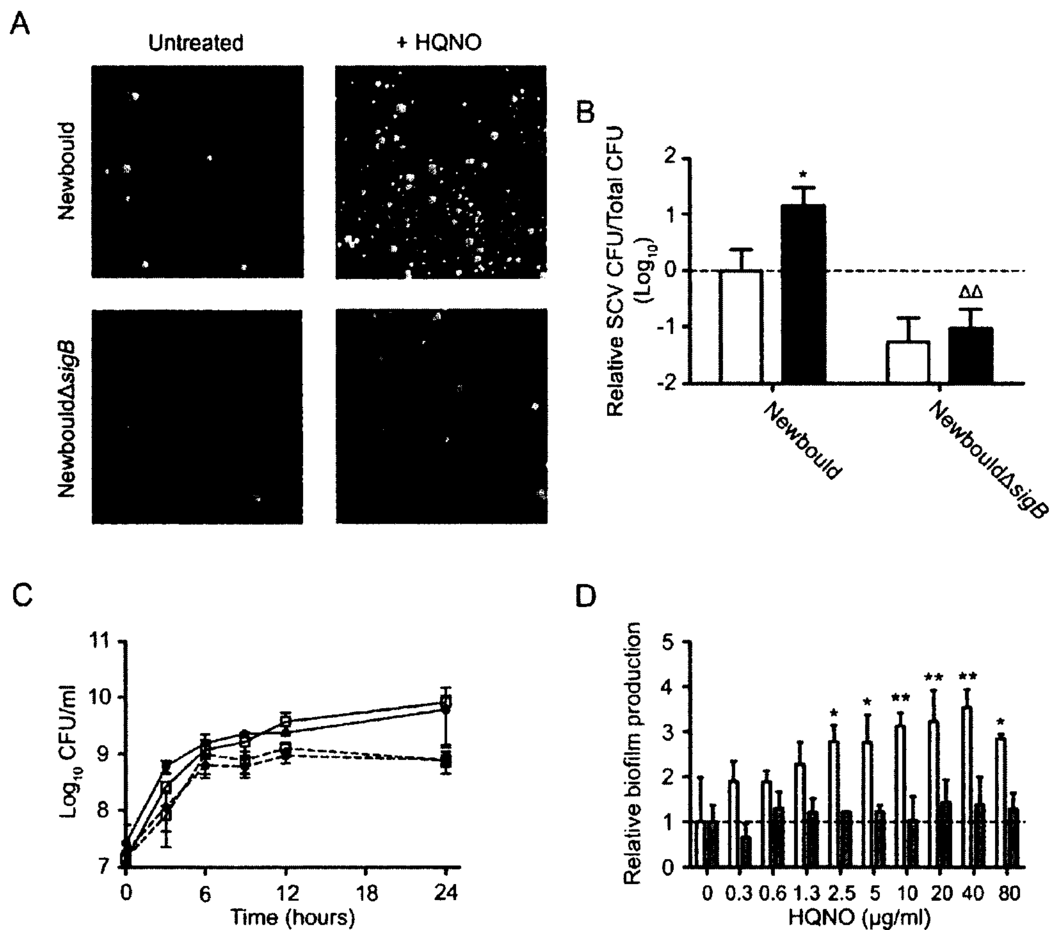


FIGURE 3. SigB is involved in HQNO-mediated emergence of SCVs and biofilm production. (A) Pictures show SCV colonies grown on agar containing a selective concentration of gentamicin following or not an overnight exposure to 10 µg/ml of HQNO for strains Newbould and NewbouldΔ*sigB*. (B) Relative number of SCV CFUs recovered after 18 h of growth for strains Newbould and NewbouldΔ*sigB* in the presence (black bars) or not (open bars) of 10 µg HQNO/ml. Results are normalized to unexposed Newbould (dotted line). Data are presented as means with standard deviations from at least three independent experiments.

Significant differences between unexposed and HQNO-exposed conditions (*, $P < 0.05$), and between strains in the same experimental condition (Δ, $P < 0.05$) were revealed by a one-way ANOVA with tuckey's post test. (C) Growth curves of Newbould (□) and NewbouldΔsigB (●) exposed (dotted lines) or not (solid lines) to 10 µg/ml of HQNO. (D) Relative biofilm formation as a function of the concentration of HQNO for strains Newbould (open bars) and Newbould*ΔsigB* (grey bars). Results are normalized to the unexposed condition for each strain (dotted line). Data are presented as means with standard deviations from two independent experiments. Significant differences between Newbould and Newbould*ΔsigB* for each concentration of HQNO are shown (*, $P < 0.05$; **, $P < 0.01$; two-way ANOVA with bonferroni's post test).

SigB is involved in HQNO-mediated emergence of SCVs and biofilm production

Strains Newbould and Newbould*ΔsigB* were used to determine whether SigB is involved in the emergence of SCVs and biofilm production under an exposure to HQNO. Fig. 3A illustrates the ability of HQNO (10 µg/ml, overnight) to favor the emergence of the SCV phenotype only in a $sigB^{+}$ background. HQNO significantly increased the presence of SCVs in strain Newbould, but not in Newbould*ΔsigB* (Fig. 3B). This result was confirmed with strains SH1000 and 8325-4 (data not shown), which are isogenic strains with a functional and dysfunctional SigB system, respectively [36]. Fig. 3C demonstrates that the presence of HQNO significantly inhibits the growth of both Newbould and Newbould*ΔsigB* ($P < 0.05$ at 24h of growth for both; two-way ANOVA followed by a Bonferroni's post test). However, the ability of HQNO to increase biofilm formation was observed with strain Newbould, but not with Newbould*ΔsigB* (Fig.3D). These results suggest that, even if the inhibition of growth caused by HQNO is not influenced by SigB (Fig. 3C), HQNO-mediated emergence of SCVs and biofilm production is triggered by a SigB-dependent mechanism (Fig. 3D).

SigB and *agr* activities are modulated by an exposure to HQNO

Fig. 4 shows qPCR measurements of the expression of the genes *asp23*, *fnbA*, *hld* (RNAIII), *hla*, *sarA* and *gyrB* at the exponential growth phase for strains Newbould and NewbouldΔ*sigB* exposed or not to HQNO. The expression of *asp23* and *fnbA* was evaluated in order to verify the hypothesis that SigB is activated during HQNO exposure. The gene *asp23* is a well-known marker for SigB activity as for the gene *fnbA*, although the transcription of the latter is not exclusively influenced by SigB [15, 19, 22, 37]. Fig. 4A and 4B show that HQNO at 10 µg/ml induced SigB activity in strain Newbould, as revealed by significant increases of *asp23* and *fnbA* expression. The effect of HQNO on the expression of *asp23* and *fnbA* was further confirmed with the sequenced strain Newman (data not shown). These results suggest that SigB activity is increased by HQNO.

The activity of the *agr* system is known to be reduced in SCVs [15, 38-41]. We have thus hypothesized that HQNO exposure would repress the *agr* quorum-sensing system due to the general suppression of growth toward normal strains (likely mediated through the inhibition of the electron transport chain by HQNO [42]) but also due to the overall emergence of the SCV sub-population as seen in Fig. 1. Indeed as expected, Fig. 4C shows that exposure of Newbould and NewbouldΔ*sigB* to HQNO significantly repressed the expression of *hld* (the effector of the *agr* system).

With the increased in SigB activity and the reduced expression of *agr* observed under exposure to HQNO, it was also justified to measure the expression of the α-hemolysin gene *hla* which can be influenced by both *agr* and SigB [36, 43]. *hla* was only significantly repressed in Newbould and not in NewbouldΔ*sigB* by the presence of HQNO (Fig. 4D). Furthermore, the expression of *hla* was, in both exposed and unexposed conditions, significantly increased in NewbouldΔ*sigB* in comparison to Newbould, which confirms the negative influence of SigB on *hla* expression [36]. These results show that the expression of

hla is reduced by HQNO and that the influence of SigB on *hla* expression under HQNO exposure seems to be predominant over the *agr* system.

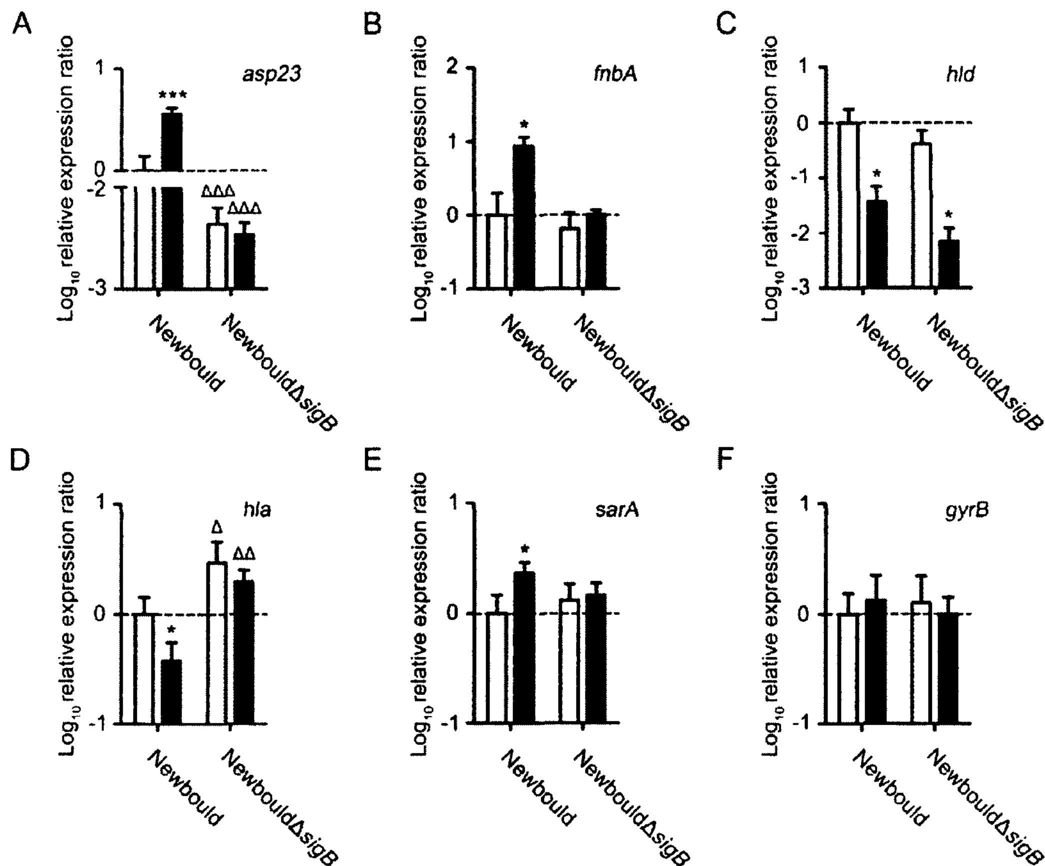


FIGURE 4. SigB and *agr* activities are modulated by an exposure to HQNO. Relative expression ratios for the genes *asp23* (A), *fnbA* (B), *hld* (C), *hla* (D), *sarA* (E) and *gyrB* (F) were evaluated by qPCR for strains Newbould and NewbouldΔ*sigB* grown to the exponential phase in the presence (black bars) or in the absence (open bars) of 10 μg/ml of HQNO. Results are normalized to unexposed Newbould (dotted line). Data are presented as means with standard deviations from at least three independent experiments. Significant differences between the unexposed and HQNO-exposed conditions (*, $P < 0.05$; ***, $P < 0.001$) and

between Newbould and NewbouldΔ*sigB* for the same experimental condition (Δ, $P < 0.05$; $\Delta\Delta$, $P < 0.01$; $\Delta\Delta\Delta$, $P < 0.001$) were revealed by one-way ANOVA followed by the tuckey's post test.

The expression level of *sarA* was also measured because of its partial dependency on SigB for expression [22, 23], and its roles in the regulation of virulence factors expression [24] and in biofilm formation [29]. Fig. 4E shows that *sarA* expression is significantly induced by HQNO in strain Newbould but not in NewbouldΔ*sigB*. The specificity of the observed modulations in gene expression was validated by monitoring the impact of HQNO on the expression of the housekeeping gene *gyrB*. The expression of *gyrB* was not modulated in the different conditions tested (Fig. 4F). These results suggest that HQNO induces the expression of *sarA* by a SigB-dependent mechanism.

Overall, these results suggest that exposure of *S. aureus* to HQNO reproduces the transcriptional signature found in SCVs [12, 15, 19, 20, 41] and stimulates biofilm production by having opposite effects on the activity of SigB (up) and *agr* (down) as well as on the expression of *sarA* (up by a SigB-dependent mechanism).

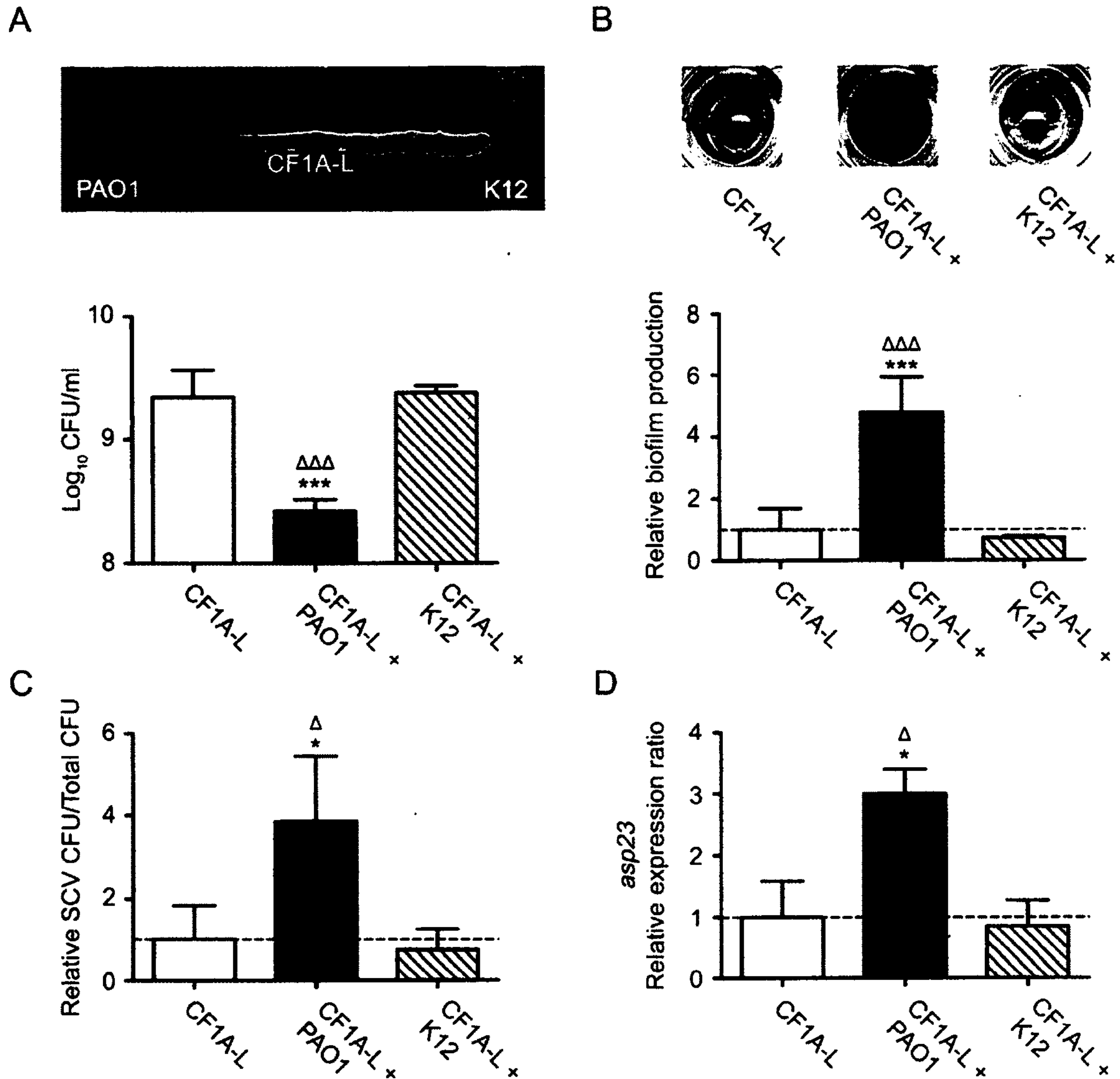


FIGURE 5. *P. aeruginosa* stimulates biofilm formation and increases the activity of SigB of a *S. aureus* CF isolate. (A) CFU/ml recovered after 48 h of growth of CF1A-L (open bar) and CF1A-L in the presence of supernatants from overnight cultures of *P. aeruginosa* PAO1 (black bar) or of *E. coli* K12 (hatched bar). The picture shows the specific inhibitory effect of *P. aeruginosa* on the growth of *S. aureus*. (B) Relative biofilm production by CF1A-L grown in the presence of supernatants from overnight cultures of *P. aeruginosa* or *E. coli*. Pictures show the biofilm formation of CF1A-L in the absence or in the presence of culture supernatants of *P. aeruginosa* or *E. coli* as detected by crystal violet staining. (C) Relative

number of SCV CFUs recovered after 6 h of growth for *S. aureus* CF1A-L in co-culture with PAO1 or K12 as determined using the double chamber co-culture model. (D) Relative expression ratios for the gene *asp23* were evaluated by qPCR for CF1A-L in co-culture with PAO1 or K12. For B, C and D, results are normalized to unexposed CF1A-L (dotted line). Data are presented as means with standard deviations from three independent experiments. Significant differences between unexposed CF1A-L and the exposed conditions (*, $P < 0.05$; ***, $P < 0.001$) and between CF1A-L exposed to PAO1 or K12 (Δ, $P < 0.05$; ΔΔΔ, $P < 0.001$) were revealed by one-way ANOVA followed by the tuckey's post test.

***P. aeruginosa* stimulates biofilm formation and increases the activity of SigB of a *S. aureus* CF isolate.** In order to ascertain that the effect of HQNO on *S. aureus* is representative of what may happen when *P. aeruginosa* and *S. aureus* are in close proximity during a co-infection, we conducted experiments in which *S. aureus* was exposed to supernatants from overnight cultures of *P. aeruginosa* as well as experiments using a double chamber co-culture model. We used the *E. coli* strain K12 in control experiments to ensure that the observed effect was specific to *P. aeruginosa* and was not only caused by the close proximity of a Gram-negative bacterium or non specific alterations of the growth medium. We used *E. coli* because it is known that this bacterium does not produce HQNO (E. Déziel, unpublished data). Fig.5A shows that *P. aeruginosa* PAO1 inhibits the growth of the *S. aureus* strain CF1A-L whereas this phenomenon was not observed with *E. coli* K12. The supernatant collected from an overnight culture of PAO1 significantly inhibited the growth of *S. aureus*. This growth inhibition was accompanied by a significant increase in biofilm production (Fig. 5B). Fig. 5C shows that when *S. aureus* CF1A-L was co-cultured with PAO1 for 6 h, significantly more SCVs were recovered than that seen when the co-culture was done with *E. coli* K12. Of striking interest, the co-cultivation of *S. aureus* CF1A-L with *P. aeruginosa* PAO1 specifically and significantly increased the expression of *asp23*. These results confirm that *P. aeruginosa* has the potential to specifically inhibit the growth, stimulate biofilm production, favor the

emergence of the SCV phenotype and increase the activity of SigB in non-SCV *S. aureus* strains.

HQNO from *P. aeruginosa* stimulates *S. aureus* biofilm production by a SigB-dependent mechanism. We used the *pqsA* and *pqsL* mutants derived from *P. aeruginosa* PA14 to further confirm the specific effect of HQNO on biofilm production by *S. aureus*. The *pqsA* mutant does not produce any 4-hydroxy-2-alkylquinolines (HAQs) at all [44, 45], whereas the *pqsL* mutant is specifically altered in HQNO biosynthesis [46]. Thus, we have used both *pqsA* and *pqsL* mutants in order to distinguish the global impact of all *P. aeruginosa* HAQs from the specific impact of HQNO on biofilm production by *S. aureus*. Fig. 6A shows that the growth of the *pqsA* and *pqsL* mutants is not impaired compared to that of the parental strain PA14, thus excluding variations in supernatant composition caused by differences in growth rates among strains. Fig. 6B shows that the supernatant from an overnight culture of *P. aeruginosa* PA14 stimulates biofilm production by *S. aureus* CF1A-L in comparison to the supernatant from the *pqsL* mutant (specific HQNO-minus strain). The effect of different doses of supernatants from overnight cultures of *P. aeruginosa* PA14, the *pqsA* mutant, the *pqsL* mutant or *E. coli* K12 on biofilm production by *S. aureus* CF1A-L is shown in Fig. 6C. While supernatants from both mutants significantly induced less biofilm production in comparison to PA14, this attenuated effect was more pronounced for the *pqsA* mutant (negative for the production of all HAQs) than the *pqsL* mutant. This result can be explained by the fact that other HAQs secreted by *P. aeruginosa*, although less potent than HQNO, can also have a growth-inhibitory activity against *S. aureus* [47]. Noteworthy, all three strains of *P. aeruginosa* stimulated biofilm production in comparison to *E. coli*, suggesting that other *P. aeruginosa* exoproducts can indeed stimulate biofilm production by *S. aureus*.

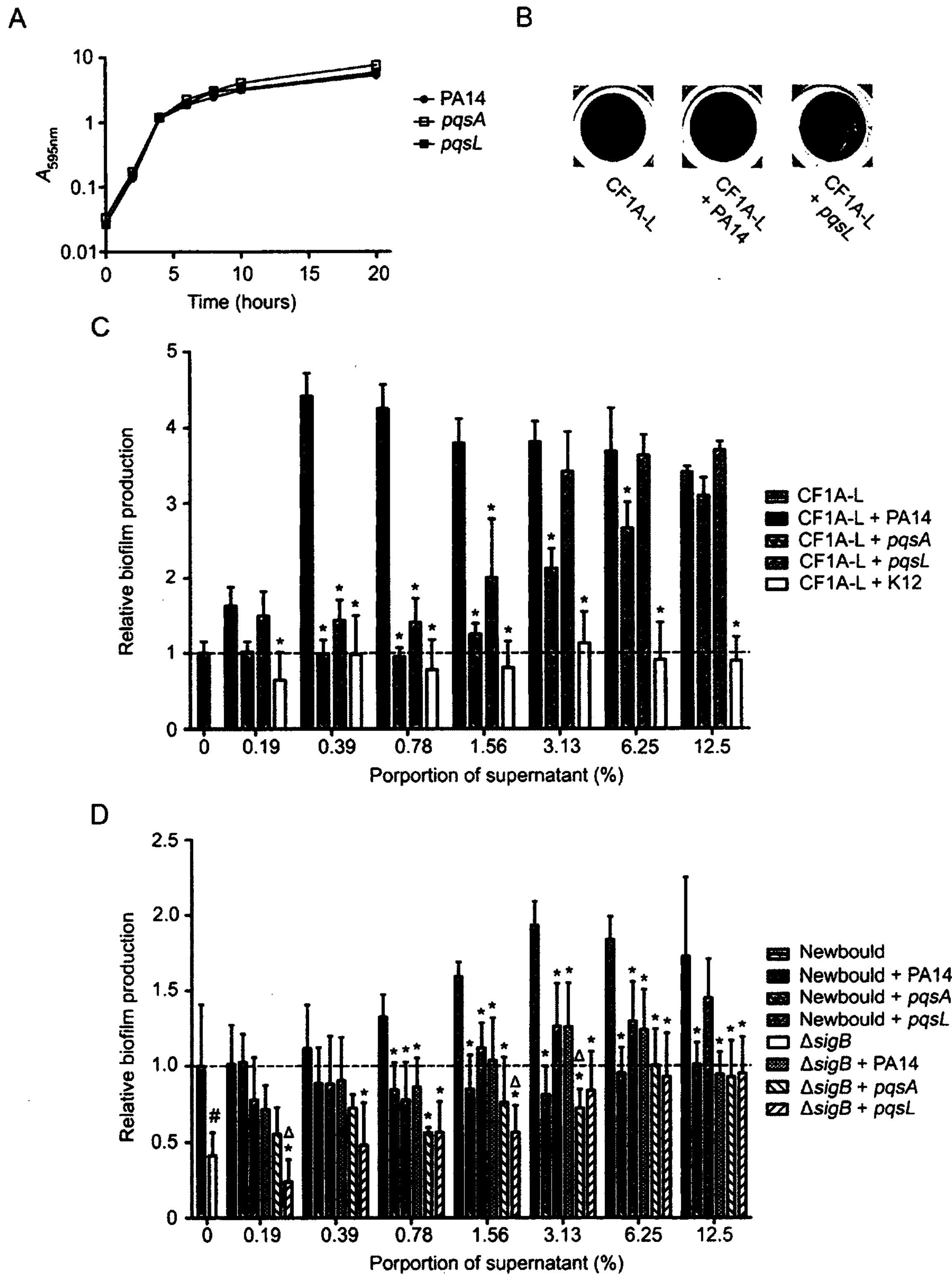

A
B
C
D
10
1
0.1
0.01
0
5
10
15
20
Time (hours)
A_{595nm}
PA14
pqsA
pqsL
CF1A-L
CF1A-L + PA14
CF1A-L + pqsL
Relative biofilm production
Porportion of supernatant (%)
0.19
0.39
0.78
1.56
3.13
6.25
12.5
CF1A-L + pqsA
CF1A-L + K12
Newbould
Newbould + PA14
Newbould + pqsA
Newbould + pqsL
ΔsigB
ΔsigB + PA14
ΔsigB + pqsA
ΔsigB + pqsL

FIGURE 6. HQNO from *P. aeruginosa* stimulates biofilm production of *S. aureus* strains by a SigB-dependent mechanism. (A) Growth curves of *P. aeruginosa* strain PA14 and the *pqsA* and *pqsL* mutants. (B) Pictures show relative biofilm production of CF1A-L in the absence or in the presence of supernatants from overnight cultures of *P. aeruginosa* PA14 or the *pqsL* mutant as determined by crystal violet staining. (C) Relative biofilm production by *S. aureus* CF1A-L as a function of the proportion of supernatant from overnight cultures of *P. aeruginosa* PA14, the *pqsA* mutant, the *pqsL* mutant or *E. coli* K12. Results are normalized to unexposed CF1A-L (dotted line). Significant differences between CF1A-L+PA14 and the other conditions for each proportion of supernatant are shown (*, $P < 0.05$; two-way ANOVA with Bonferroni's post test). (D) Relative biofilm production by *S. aureus* strains Newbould and NewbouldΔ*sigB* as a function of the proportion of supernatant from overnight cultures of *P. aeruginosa* PA14, the *pqsA* or the *pqsL* mutant. Significant differences between Newbould + PA14 and the other conditions for each proportion of supernatant (*, $P < 0.05$; two-way ANOVA with Bonferroni's post test), and between NewbouldΔ*sigB* + PA14 and Newbould Δ*sigB* + the *pqsA* or the *pqsL* mutant (Δ, $P < 0.05$; two-way ANOVA with Bonferroni's post test) are shown. The significant difference between untreated Newbould and NewbouldΔ*sigB* is also shown (#, $P < 0.05$; unpaired *t*-test). Data are presented as means with standard deviations from at least three independent experiments.

Fig. 6D confirms that HQNO from the supernatant of strain PA14 stimulates biofilm production by a SigB-dependent mechanism. The increase in biofilm production observed when *S. aureus* Newbould is in contact with the supernatant from PA14 is significantly higher than that seen with supernatants from the *pqsA* and *pqsL* mutants. Surprisingly, both mutants did not significantly stimulate biofilm production by Newbould as that observed for CF1A-L, suggesting that differences between *S. aureus* strains may exist in respect to their response to the presence of non-HQNO exoproducts. As expected, biofilm production by NewbouldΔ*sigB* in contact with supernatants from the three *P. aeruginosa* strains was significantly inferior to that observed using the PA14 supernatants with strain Newbould. Moreover, supernatants

from PA14 generally did not significantly stimulate biofilm production by NewbouldΔ*sigB* in comparison to supernatants from *pqsA* and *pqsL* mutants, which confirms that SigB is involved in HQNO-mediated *S. aureus* biofilm production. Overall, the results of this section support the hypothesis that HQNO from *P. aeruginosa* stimulates biofilm production by *S. aureus* through a SigB-dependent mechanism.

DISCUSSION

We found that the *P. aeruginosa* exoproduct HQNO increases the production of biofilm by *S. aureus*. The effects on biofilm production, as well as on growth, were only seen on normal strains whereas the already high biofilm formation and slow growth rate of SCVs were not altered by the presence of HQNO. It is known that HQNO specifically inhibits electron transport in Gram-positive bacteria [42] and its immediate action toward normal strains is likely to reduce ATP production and growth to a similar level to that observed in SCVs. Interestingly, our results also indicate that HQNO provokes a sustained stimulatory effect on the production of biofilms by *S. aureus*. We indeed found that a pre-treatement of *S. aureus* with HQNO still led to a subsequent increase in biofilm formation even after HQNO removal. This sustained effect is probably associated with the increased proportion of the sub-population of SCVs resulting from HQNO exposure. An exposure of *S. aureus* to HQNO may thus, in addition to its immediate effect, favor the emergence of SCVs having a long-term impact on biofilm formation.

Aminoglycosides are also known to favor the emergence of SCVs [12] and are often used in CF patient care [1]. Interestingly, a synergistic effect between HQNO and tobramycin for the formation of *S. aureus* SCVs was previously observed by Hoffmann *et al.* [2]. It is thus possible that the administration of aminoglycosides to CF patients co-infected with both *S.*

aureus and *P. aeruginosa* further increases the formation of biofilm by *S. aureus*. Besides, it is well known that the abnormal function of the CF transmembrane conductance regulator (CFTR) protein in CF patients has profound consequences on the airway physiology and it will be of great interest to determine whether other parameters related to the CF airways influence the emergence of SCVs and the production of biofilms by *S. aureus*. The expression of virulence factors in *S. aureus* is indeed controlled by diverse and complex regulatory networks in a time- and environment-dependent manner, being influenced for example by ionic forces, pH and O_2 [48]. Consequently, it is likely that *S. aureus* specifically responds to the particular environment of CF airways. Whether this response is SigB-dependent and will lead to the emergence of SCVs and biofilm production remains to be determined.

Naturally-occurring mutations altering the activity of virulence regulators in *S. aureus* have been previously reported [36, 49-52]. Our results suggest that the inactivation of *sigB* will importantly influence the outcome of the HQNO-mediated interaction between *P. aeruginosa* and *S. aureus*. We are currently studying *S. aureus* isolates from CF patients co-infected with *P. aeruginosa* which are not influenced by the presence of *P. aeruginosa*. This, in addition to the observation that differences between *S. aureus* strains exist relative to their response to HAQs (Fig. 6C and D), suggest that *S. aureus* strains isolated from CF patients may adapt or evolve toward a long-term coexistence with *P. aeruginosa*. Whether this involves mutations in *sigB* or any other genes encoding regulators is now under investigation and will greatly help to understand the dynamic behavior and the adaptation of *S. aureus* in response to the CF airway environment as well as to the presence of *P. aeruginosa*.

The effect of HQNO on the regulators SarA, *agr* and SigB suggests that several virulence factors should be influenced by the presence of HQNO. A concomitant activation of SigB and repression of *agr* should result in the up-regulation of a variety of cell-surface proteins (such as FnBA) involved in adhesion to host tissues, and in the repression of several exotoxins (such

as α-hemolysin, Hla) and proteolytic enzymes [22, 25]. It is then tempting to speculate that the presence of HQNO will prevent *S. aureus* from disseminating and will rather favor tissue colonization, biofilm production and invasion of host cells. It has indeed been suggested that *S. aureus* FnBPs mediates cellular invasion [53, 54] whereas the capacity of the bacterium to remain intracellular is helped by the repression of *hla* [55]. Accordingly, we showed that an exposure of *S. aureus* to HQNO up-regulates the expression of *fnbA* and represses the expression of *hla*. However, whether or not HQNO and *P. aeruginosa* increase the invasion of host cells by *S. aureus* remains to be confirmed. Interestingly, O'Neil *et al.* [32] have recently demonstrated that the FnBPs are also involved in the *ica*-independent mechanism of biofilm formation. It is thus possible that FnBPs are directly responsible for the observed HQNO-mediated SigB-dependent increase in biofilm production and, more specifically, FnBPA which is under the control of SigB for expression [15, 19, 22, 37]. As such, the FnBPs would represent the main effectors for both biofilm formation and cellular invasion in *S. aureus* SCVs.

HQNO may be one of several bacterial exoproducts influencing *S. aureus* during polymicrobial infections. Our results and those of Machan *et al.* [47] suggest that other HAQs may also affect *S. aureus*, although not as efficiently as HQNO. Moreover, it is known that other *P. aeruginosa* exoproducts such as pyocyanin have an inhibitory activity against the electron transport chain of *S. aureus* [13]. Loss of pyocyanin production has been associated with mutations in the *pqsA-E* genes [45, 56], which may provide an additional explanation for the different effects of the *pqsA* and *pqsL* mutants we have observed on the growth (data not shown) and biofilm formation of *S. aureus* (Fig. 6C). Furthermore, Qazi *et al.* [7] found that an *N*-acyl-homoserine-lactone from *P. aeruginosa* antagonizes quorum sensing and virulence gene expression in *S. aureus*. More precisely, it was shown that the 3-oxo-C_{12}-HSL interacts with the cytoplasmic membrane of *S. aureus* and down-regulates both *sarA* and *agr* expression. Although we also observed here a down-regulation of *agr*, the HQNO-mediated up-regulation of *sarA* suggests further complexity in the response of *S. aureus* to *P.*

aeruginosa exoproducts. It is possible that the outcome of the *S. aureus-P. aeruginosa* interaction is dependent on the amount and the types of exoproducts secreted by the specific strain of *P. aeruginosa* interacting with *S. aureus*.

It should be kept in mind that the diversity of diffusible bacterial signals and their role in interspecies communication are just beginning to be appreciated [9] and that potentially numerous other bacterial species may be interacting with *S. aureus*, especially during infectious diseases. It is then likely that *S. aureus* interacts with other bacterial genus than *Pseudomonas* during infection of the airways of CF patients. As an example, the CF pathogen *Burkholderia cepacia* also produces *N*-acylhomoserine lactones [57] and some *Burkholderia* species are able to synthesize HAQ analogues [58]. Nevertheless, the observation that *P. aeruginosa* favors the emergence of SCVs and biofilm production by *S. aureus* is likely to have a significant clinical impact. The clinical consequences may actually surpass the previously anticipated formation of aminoglycoside-resistant SCVs by Hoffman *et al.* [2]. Persistence of bacteria in chronic infections has been associated with biofilm production [1, 59] and biofilms are known to confer protection from host defenses and antibiotic treatments at large [34, 60]. In the cystic fibrosis context, where obstructive infections worsen the health prognosis of patients, the clinical significance of biofilm production by normal *S. aureus* and SCV strains will need to be further investigated.

CONCLUSIONS

This study strongly supports the hypothesis that *P. aeruginosa* influences the pathogenicity of *S. aureus* by producing HQNO, which favors the acquisition of the SCV phenotype through the activation of the stress- and colonization-related *S. aureus* alternative sigma factor B. Although several *P. aeruginosa* exoproducts may potentially influence *S. aureus*, our

observations with pure HQNO were confirmed and supported by experiments using whole supernatants from two *P. aeruginosa* strains as well as mutants unable to produce HQNO. Considering that biofilms and SCVs are both suspected to play a role in chronic infections of CF airways, the observation that *P. aeruginosa* increases the emergence of SCVs and biofilm formation by *S. aureus* may influence the patient health prognosis. New therapeutic strategies should aim at preventing interspecies interactions and the development of specific phenotypes such as biofilm-producing SCVs in order to reduce the likelihood of chronic infections.

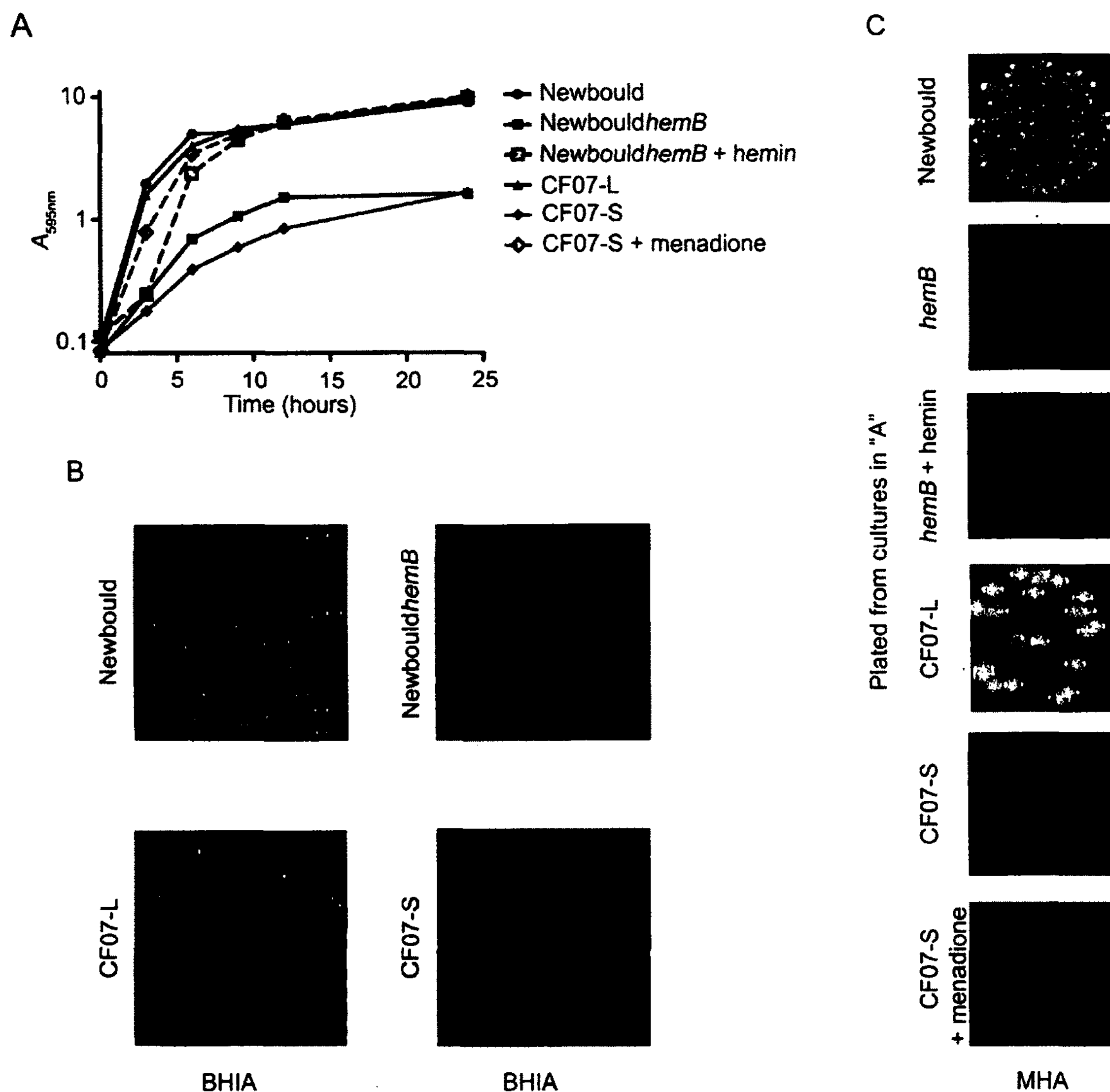
METHODS

Bacterial strains and growth conditions

The relevant characteristics of the strains used in this study are shown in Table 1. *Staphylococcus aureus* ATCC 29213, Newman and Newbould were used as representatives of prototypical control strains. NewbouldΔ*sigB* and Newbould*hemB*, in which the genes *sigB* or *hemB* had been disrupted by the *ermA* cassette [15, 17], were used to evaluate the importance of SigB in a prototypical background and to generate a stable SCV, respectively. CF03-L/CF03-S, CF07-L/CF07-S and CF1A-L/CF1D-S are related pairs of strains co-isolated from CF patients, which respectively have a normal and a SCV phenotype. The genetic relatedness of each strain among the pairs was confirmed by the analysis of multiple loci with a variable number of tandem repeats (see below). Except where otherwise stated, *S. aureus* strains were grown in brain heart infusion (BHI) broth (BD, ON, Canada). The use of BHI to study our SCV strains as well as in the experiments involving quantification of SCVs is validated in the Additional file 1. *Pseudomonas aeruginosa* PAO1 [61], PA14 [62], the PA14-derived *pqsA* and *pqsL* mutants [44, 46] and *Escherichia coli* K12 were grown in trypticase soy broth (TSB) (BD, ON, Canada).

TABLE 1. Bacterial strains used in this study.

Strains	Relevant characteristics	Auxotrophism	References
S. aureus strains			
ATCC 29213	Laboratory strain, normal	-	-
Newman ATCC 25904	Laboratory strain, normal	-	-
Newbould ATCC 29740	Laboratory strain, normal	-	-
NewbouldΔ*sigB*	Newbould Δ*sigB*::*emrA*; Erm^R	-	[15]
Newbould*hemB*	Newbould *hemB*::*ermA*; Erm^R	Hemin	[17]
CF03-S	SCV strain isolated from a CF patient	Menadione	[15]
CF03-L	Normal strain co-isolated with CF03-S	-	This study
CF07-S	SCV strain isolated from a CF patient	Menadione	[15]
CF07-L	Normal strain co-isolated with CF07-S	-	This study
CF1D-S	SCV strain isolated from a CF patient	Unknown	This study
CF1A-L	Normal strain co-isolated with CF1D-S	-	This study
P. aeruginosa strains			
PAO1	Laboratory strain	-	[61]
PA14	Clinical strain, Rif^R	-	[62]
pqsA	PA14 *pqsA*::Tn*phoA*; Rif^R, Km^R	-	[44]
pqsL	PA14 Δ*pqsL*; Rif^R	-	[46]
E. coli strains			
K12	Laboratory strain	-	-



ADDITIONAL FILE 1. Validation of the use of BHI as the growth medium to induce and study SCVs. (A) Growth curves expressed in absorbance at 595 nm for the strains Newbould, Newbould*hemB*, CF07-L and CF07-S. The growth of Newbould*hemB* and CF07-S was supplemented or not with 5 μg/ml of hemin and 1 μg/ml of menadione, respectively. Results show that SCVs present their slow-growth phenotype in BHI unless supplemental hemin or menadione is added to the broth. (B) Pictures of colonies from strains Newbould, Newbould*hemB*, CF07-L and CF07-S grown on BHI agar for 16 hours. Results show that SCVs retain their slow-growth phenotype on BHIA in comparison to normal strains. (C)

Appearance of the colonies obtained from the cultures shown in A at the 12-h time point and plated on Mueller-Hinton agar (MHA) for 36 hours. Results show that normal strains grow efficiently on MHA whereas SCVs taken from BHI broth (cultures shown in A and supplemented or not with hemin or menadione) still present their slow-growth phenotype once plated back on MHA.

Multiple-locus variable-number of tandem repeat analysis (MVLA) of strains co-isolated from CF patients

The relatedness of each of the co-isolated strains within the pairs CF03-L/CF03-S, CF07-L/CF07-S and CF1A-L/CF1D-S was confirmed by MVLA as described by Sabat *et al.* [63]. The strains of each pair had identical MVLA patterns.

Growth curves

S. aureus overnight cultures were used at an A_{595nm} of 0.1 to inoculate BHI broths supplemented or not with 10 µg/ml of HQNO (Axxora, CA, USA). Cultures were then incubated at 35°C/225 RPM and samples were taken at different time points for determination of CFU by spreading 10-fold dilutions on trypticase soy agar (TSA) plates (BD, ON, Canada). Plates were incubated at 35°C for 24 and 48 h for normal and SCV strains, respectively. For the growth curves of *P. aeruginosa* PA14 and the *pqsA* and *pqsL* mutants, overnight cultures were used to inoculate TSB. Cultures were then incubated at 35°C/225 RPM and samples were taken at specified time points in order to evaluate their turbidity at A_{595nm}.

Quantification of SCVs

We have quantified SCVs by taking advantage of their reduced susceptibility to aminoglycosides as described elsewhere with few modifications [20, 64, 65]. A 1:100 dilution of overnight broth cultures was used to inoculate BHI broths supplemented or not with 10

µg/ml of HQNO. Cultures were incubated 18 h and then adjusted to an A_{595nm} of 2.0 in PBS at 4°C. Determination of SCV CFUs was done by serial dilution plating. SCV counts were obtained by plating on TSA containing gentamicin (Sigma-Aldrich, ON, Canada) at 4 µg/ml followed by an incubation of 48 h at 35°C. As shown in Additional file 2, this concentration of gentamicin is selective for SCVs as it allows the growth of SCVs, but not that of normal strains. The frequency of SCVs is defined as the number of SCVs per total CFU counts on antibiotic-free TSA. The pinpoint colonies detected by this gentamicin-plate method were confirmed to be SCVs by streaking several of them on TSA plates (See Additional file 3). We have also evaluated the auxotrophism (as described below) of several HQNO-induced SCVs generated from strains CF1A-L and CF07-L in order to further validate the ability of this technique to detect typical SCVs (see Additional file 4).

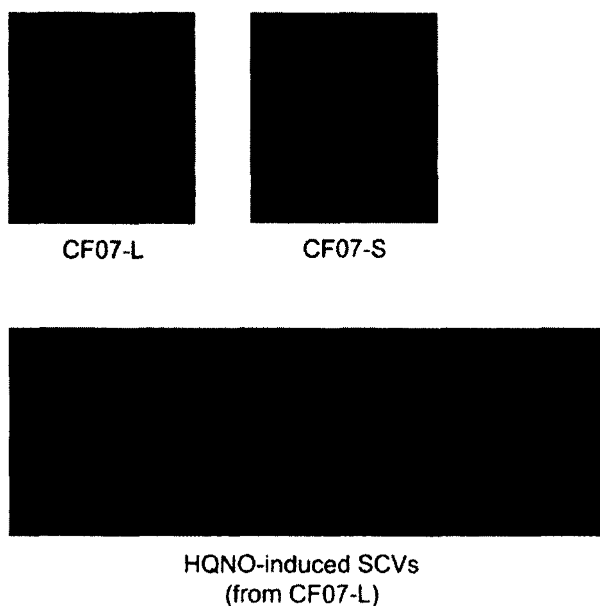
ADDITIONAL FILE 2. Minimal inhibitory concentrations (MICs) of gentamicin for the studied strains.

TABLE 2. Minimal inhibitory concentrations (MICs) of gentamicin for the studied strains

Strains	µg/ml
ATCC29213	0.5-1
Newman	1
Newbould	0.5-1
Newbould∆*sigB*	0.5-1
Newbould*hemB*	8
CF03-S	8
CF03-L	0.5-1
CF07-S	8
CF07-L	1
CF1D-S	8
CF1A-L	1

Antibiotic susceptibility

The minimal inhibitory concentrations (MICs) of gentamicin for all strains were determined by a broth microdilution technique, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines [66], except that the incubation period was extended to 48 h and that the medium used was BHI in order to allow SCVs to reach maximal growth.



ADDITIONAL FILE 3. Appearance of HQNO-induced SCVs selected on gentamicin-containing agar and streaked back on TSA plates. Pictures are showing CF07-L, CF07-S and HQNO-induced SCVs selected on gentamicin-containing agar and streaked back on TSA plates. The bottom pictures show streaks of three isolated SCVs on TSA plates. Many more SCVs were similarly tested and our results showed that at least 85% of the SCVs isolated from gentamicin plates were keeping their slow-growth phenotype when subsequently grown on TSA without gentamicin.

Auxotrophism of SCVs.

In the context of SCVs, auxotrophism is defined as the requirement of specific compounds in order to regain a normal growth phenotype [41]. An agar diffusion method was used to characterize the auxotrophism of SCVs using hemin or menadione (10 µg each/well) on an inoculated Mueller-Hinton agar (MHA) plate. Thymidine at 1.5 µg/well was also tested as previously described [67]. Auxotrophy for specific supplements was detected by a zone of normal growth surrounding the well after 18 h of incubation at 35°C. The photography of the Additional file 5 shows the normal growth of Newbould*hemB* in proximity of a well loaded with hemin as an example of a positive auxotrophism result.

ADDITIONAL FILE 4. Auxotrophism found among HQNO-induced SCVs.

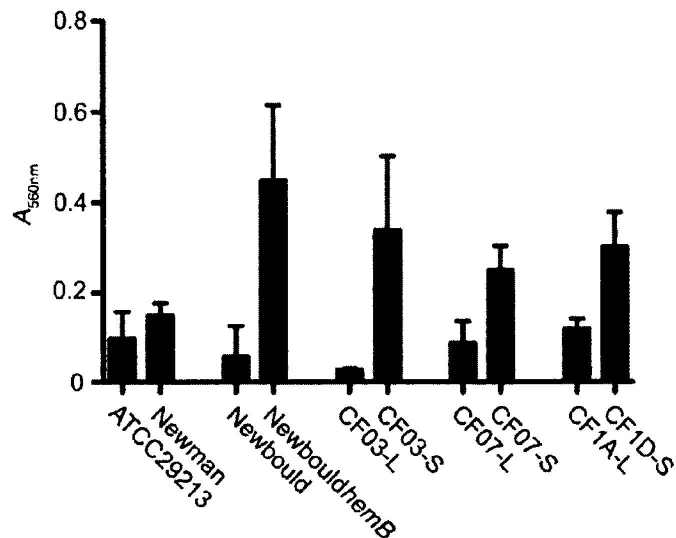
TABLE 3. Auxotrophism of SCVs recovered after an exposure to HQNO

Auxotrophism	Number of SCV colonies for each strain exposed to HQNO	
	CF07-L	CF1A-L
Hemin	0	0
Menadione	15	12
Thymidine	0	0
Unknown	1	1

ADDITIONAL FILE 5. Growth of Newbould*hemB* in proximity of a well loaded with hemin. Growth of Newbould*hemB* in proximity of a well loaded with hemin as an example of a positive auxotrophism result. The auxotrophism of Newbould*hemB* for hemin is seen by observing normal growth only within the diffusion zone of a well loaded with hemin.

Preparation of supernatants from *P. aeruginosa* and *E. coli* strains

Overnight cultures were used to inoculate TSB at a dilution of 1:100. Cultures were then incubated 20 h at 35°C/225 RPM before collecting the culture supernatants by centrifugation. Similar culture conditions were previously shown to allow maximal production of HQNO by *P. aeruginosa* PAO1 [68]. The supernatants were then filter-sterilized using 0.22 μ pore size (Millipore, MA, USA) and used immediately. The sterility of the supernatants was confirmed by plating samples on TSA plate.



ADDITIONAL FILE 6. Non-normalized absorbance values at 560 nm representing biofilm production for each of the strains used in Fig. 2. Non-normalized absorbance values at 560 nm representing biofilm production for each of the strains used in Fig. 2. Results show that strains vary in their relative production of biofilms but that for each related pairs of normal and SCV strains, SCV counterparts always produce more biofilm than their respective normal strains.

Biofilm formation

For studying the effect of HQNO on biofilm production by *S. aureus*, three colonies grown on blood agar plates were used to inoculate BHI broths containing 0.25% glucose with or without 10 μg/ml of HQNO and cultures were incubated for 18 h. These cultures were used to adjust an appropriate volume of BHI-0.25% glucose to 0.5 Mcfarland for transfer into wells of a flat-bottom polystyrene microtiter plate containing half volume of the same medium with or without HQNO (final concentration 10 μg/ml). For experiments evaluating the effect of culture supernatants from *P. aeruginosa* and *E. coli* on *S. aureus* biofilm production, a *S. aureus* 0.5 Mcfarland suspension was prepared in BHI-0.5% glucose and transferred into wells

of a microtiter plate containing half the volume of the supernatant to be tested. The plates were incubated at 35°C for 48h. The supernatant was then discarded and the wells were delicately washed three times with 200 µl of PBS. The plates were dried, stained for 30 min with crystal violet, washed twice with 200 µl of water and allowed to dry again. A volume of 200 µl of 95% ethanol was added to each well and plates were incubated at room temperature for 1 h with frequent agitation. The absorbance of each well was then measured at 560 nm using a plate reader (Bio-Tek Instruments). The biofilm formation of each culture tested was evaluated in four replicates. The A_{560nm} values (non-normalized data) representing the biofilm production for each of the strains used in Fig. 2 can be seen in the Additional file 6.

Quantitative PCR (qPCR).

In order to evaluate the effect of HQNO (10 µg/ml) on *S. aureus* gene expression, overnight cultures were used to inoculate broth at an A_{595nm} of 0.1. Bacteria were then grown until the unexposed control culture reached an A_{595nm} between 0.9 and 1.0. Bacteria were collected and treated with RNAprotect (QIAGEN, ON, Canada). RNA was extracted from the cell pellets after treatment with lysostaphin (Sigma-Aldrich) (200 µg/ml, 1 h) using the RNeasy Mini kit and the RNase-free DNase set (QIAGEN). A second DNase treatment was also done with the DNA-free kit (Applied Biosystems/Ambion, CA, USA). One µg of total RNA was reverse transcribed with 0.5 mM deoxynucleotide phosphate, 50 ng of random hexamers and 200 U of Invitrogen Superscript II reverse transcriptase, according to the manufacturer's recommendations (Invitrogen, ON, Canada). RNA was hydrolyzed and the cDNAs were purified with the QIAquick PCR purification kit (QIAGEN). One microliter of the cDNA preparation was amplified on the Stratagene MX3000P Real-Time PCR instrument with the Jump Start Taq DNA polymerase (Sigma-Aldrich), SYBR Green and 100nM of the following primers:

asp23-RT-FWD 5'-TCGCTGCACGTGAAGTTAAA-3',
asp23-RT-REV 5'-CAGCAGCTTGTTTTTCACCA-3',

fnbA268-RT-FWD 5'-ACAAGTTGAAGTGGCACAGCC-3',
fnbA341-RT-REV 5'-CCGCTACATCTGCTGATCTTGTC-3',
hld-RT-FWD 5'-TAATTAAGGAAGGAGTGATTTCAATG-3'
hld-RT-REV 5'-TTTTTAGTGAATTTGTTCACTGTGTC-3'
hla-RT-FWD 5'-AATGAATCCTGTCGCTAATGCCGC-3'
hla-RT-REV 5'-CTGAAGGCCAGGCTAAACCACTTT-3'
sarA-RT-FWD 5'-CAAACAACCACAAGTTGTTAAAGC-3'
sarA-RT-REV 5'-TGTTTGCTTCAGTGATTCGTTT-3'
16SrRNA-RT-FWD 5'- TCGTTTAACACGTTTAGGTTCA-3',
16SrRNA-RT-REV 5'- GAACTGTATCAGTTGGTTTCGCAC-3',
gyrB-RT-FWD 5′-GGTGCTGGGCAAATACAAGT-3′,
gyrB-RT-REV 5′-TCCCACACTAAATGGTGCAA-3′.

Reaction mixtures were denatured for 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 60°C and 1 min 30 s at 72°C. Dissociation and standard curves were obtained to insure the specificity and the efficiency of reactions. cDNA synthesis reactions without reverse transcriptase were also routinely carried out. The relative expression ratios were calculated by using the cycle threshold (C_t) of the 16S RNA or *gyrB* of each condition as the calibrator (*n*-fold expression = $2^{-\Delta C_t}$, where ΔC_t represents the difference between the C_t of the gene studied and the C_t of the 16S RNA or *gyrB* for each condition).

Double chamber co-culture model.

Overnight cultures of *S. aureus, E.coli* and *P. aeruginosa* in TSB were used to inoculate bottom (*S. aureus, E. coli* or *P. aeruginosa*) or top (*S. aureus*) chambers of 0.4-µm pore polycarbonate membrane inserts (Transwell [Corning, MA, USA]). *S. aureus* was inoculated at an A_{595nm} of 0.01, whereas *P. aeruginosa* or *E. coli* were inoculated at an A_{595nm} of 0.1. The cultures were incubated at 35°C/80 RPM for 6 h and samples were taken for SCV enumeration

and total CFU counts as well as for RNA extraction. No bacterial cross-contamination was detected by culture plating up to at least 9 h of incubation.

Statistical analysis

One-way analysis of variance followed by Dunnett's multiple comparisons test or Tukey's multiple comparisons test were used when several conditions or strains were compared at the same time whereas unpaired *t*-tests were used when only two conditions were compared. Two-way ANOVA with Bonferroni's post tests were used to compare the response of different strains and/or different conditions as a function of the concentration of HQNO or bacterial culture supernatants. Statistical analyses of qPCR data were done on mean ΔC_t. CFU counts or SCV frequencies were transformed in based-10 logarithm values before being used for statistical analyses that were carried out with the GraphPad Prism Software (v.5.00). Statistical tests used for the analysis of each experiment are specified in figure legends.

AUTHOR'S CONTRIBUTIONS

GM, DLS and AEA carried out the experiments. GM, DLS, ED, AMC, EHF, SM and FM designed and conceived the study. GM and FM wrote the paper. All authors read and approved the final manuscript.

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REFERENCES

1. Lyczak JB, Cannon CL, Pier GB: **Lung infections associated with cystic fibrosis**. *Clin Microbiol Rev* 2002, **15**(2):194-222.
2. Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI: **Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 2006, **103**(52):19890-19895.
3. Harrison F: **Microbial ecology of the cystic fibrosis lung**. *Microbiology* 2007, **153**(Pt 4):917-923.
4. Brogden KA, Guthmiller JM, Taylor CE: **Human polymicrobial infections**. *Lancet* 2005, **365**(9455):253-255.
5. Duan K, Dammel C, Stein J, Rabin H, Surette MG: **Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication**. *Mol Microbiol* 2003, **50**(5):1477-1491.

6. Mashburn LM, Jett AM, Akins DR, Whiteley M: ***Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture.** *J Bacteriol* 2005, **187**(2):554-566.
7. Qazi S, Middleton B, Muharram SH, Cockayne A, Hill P, O'Shea P, Chhabra SR, Camara M, Williams P: **N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in *Staphylococcus aureus*.** *Infect Immun* 2006, **74**(2):910-919.
8. Riedel K, Hentzer M, Geisenberger O, Huber B, Steidle A, Wu H, Hoiby N, Givskov M, Molin S, Eberl L: **N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms.** *Microbiology* 2001, **147**(Pt 12):3249-3262.
9. Ryan RP, Dow JM: **Diffusible signals and interspecies communication in bacteria.** *Microbiology* 2008, **154**(Pt 7):1845-1858.
10. Weaver VB, Kolter R: ***Burkholderia* spp. alter *Pseudomonas aeruginosa* physiology through iron sequestration.** *J Bacteriol* 2004, **186**(8):2376-2384.
11. Stoodley P, Sauer K, Davies DG, Costerton JW: **Biofilms as complex differentiated communities.** *Annu Rev Microbiol* 2002, **56**:187-209.
12. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G: **Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections.** *Nat Rev Microbiol* 2006, **4**(4):295-305.
13. Biswas L, Biswas R, Schlag M, Bertram R, Gotz F: **Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*.** *Appl Environ Microbiol* 2009, **75**(21):6910-6912.
14. Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, Proctor RA, Peters G: **Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis.** *J Infect Dis* 1998, **177**(4):1023-1029.
15. Moisan H, Brouillette E, Jacob CL, Langlois-Begin P, Michaud S, Malouin F: **Transcription of virulence factors in *Staphylococcus aureus* small-colony variants**

isolated from cystic fibrosis patients is influenced by SigB. *J Bacteriol* 2006, **188**(1):64-76.

16. Sadowska B, Bonar A, von Eiff C, Proctor RA, Chmiela M, Rudnicka W, Rozalska B: **Characteristics of *Staphylococcus aureus*, isolated from airways of cystic fibrosis patients, and their small colony variants**. *FEMS Immunol Med Microbiol* 2002, **32**(3):191-197.
17. Brouillette E, Martinez A, Boyll BJ, Allen NE, Malouin F: **Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo***. *FEMS Immunol Med Microbiol* 2004, **41**(1):35-41.
18. Alexander EH, Hudson MC: **Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans**. *Appl Microbiol Biotechnol* 2001, **56**(3-4):361-366.
19. Mitchell G, Lamontagne CA, Brouillette E, Grondin G, Talbot BG, Grandbois M, Malouin F: ***Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients**. *Mol Microbiol* 2008, **70**(6):1540-1555.
20. Mitchell G, Brouillette E, Seguin DL, Asselin AE, Jacob CL, Malouin F: **A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides**. *Microb Pathog* 2009.
21. Price CW: **General stress response.** In: *Bacillus subtilis and its closest relatives; from genes to cells.* Edited by Sonenshein AL. Washington, D.C.: ASM; 2002: 369-384.
22. Bischoff M, Dunman P, Kormanec J, Macapagal D, Murphy E, Mounts W, Berger-Bachi B, Projan S: **Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon**. *J Bacteriol* 2004, **186**(13):4085-4099.
23. Deora R, Tseng T, Misra TK: **Alternative transcription factor σ^{SB} of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *sar***. *J Bacteriol* 1997, **179**(20):6355-6359.

24. Cheung AL, Zhang G: **Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family**. *Front Biosci* 2002, **7**:d1825-1842.

25. Novick RP, Geisinger E: **Quorum sensing in staphylococci**. *Annu Rev Genet* 2008, **42**:541-564.

26. Boles BR, Horswill AR: ***agr*-mediated dispersal of *Staphylococcus aureus* biofilms**. *PLoS Pathog* 2008, **4**(4):e1000052.

27. Kim JH, Kim CH, Hacker J, Ziebuhr W, Lee BK, Cho SH: **Molecular characterization of regulatory genes associated with biofilm variation in a *Staphylococcus aureus* strain**. *J Microbiol Biotechnol* 2008, **18**(1):28-34.

28. Rachid S, Ohlsen K, Wallner U, Hacker J, Hecker M, Ziebuhr W: **Alternative transcription factor σ^B is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate**. *J Bacteriol* 2000, **182**(23):6824-6826.

29. Beenken KE, Blevins JS, Smeltzer MS: **Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation**. *Infect Immun* 2003, **71**(7):4206-4211.

30. Lauderdale KJ, Boles BR, Cheung AL, Horswill AR: **Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation**. *Infect Immun* 2009, **77**(4):1623-1635.

31. O'Gara JP: ***ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus***. *FEMS Microbiol Lett* 2007, **270**(2):179-188.

32. O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, Foster TJ, O'Gara JP: **A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB**. *J Bacteriol* 2008, **190**(11):3835-3850.

33. Shanks RM, Meehl MA, Brothers KM, Martinez RM, Donegan NP, Graber ML, Cheung AL, O'Toole GA: **Genetic evidence for an alternative citrate-dependent biofilm formation pathway in *Staphylococcus aureus* that is dependent on fibronectin binding proteins and the GraRS two-component regulatory system**. *Infect Immun* 2008, **76**(6):2469-2477.

34. Stewart PS: **Mechanisms of antibiotic resistance in bacterial biofilms.** *Int J Med Microbiol* 2002, **292**(2):107-113.

35. Singh R, Ray P, Das A, Sharma M: **Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study.** *J Med Microbiol* 2009, **58**(Pt 8):1067-1073.

36. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ: **σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4.** *J Bacteriol* 2002, **184**(19):5457-5467.

37. Entenza JM, Moreillon P, Senn MM, Kormanec J, Dunman PM, Berger-Bachi B, Projan S, Bischoff M: **Role of σ^B in the expression of *Staphylococcus aureus* cell wall adhesins ClfA and FnbA and contribution to infectivity in a rat model of experimental endocarditis.** *Infect Immun* 2005, **73**(2):990-998.

38. Atalla H, Gyles C, Jacob CL, Moisan H, Malouin F, Mallard B: **Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis.** *Foodborne Pathog Dis* 2008, **5**(6):785-799.

39. Kahl BC, Belling G, Becker P, Chatterjee I, Wardecki K, Hilgert K, Cheung AL, Peters G, Herrmann M: **Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles.** *Infect Immun* 2005, **73**(7):4119-4126.

40. Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, Proctor RA, Hecker M, Engelmann S: **A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*.** *J Bacteriol* 2008, **190**(19):6351-6364.

41. Sendi P, Proctor RA: ***Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants.** *Trends Microbiol* 2009, **17**(2):54-58.

42. Lightbown JW, Jackson FL: **Inhibition of cytochrome systems of heart muscle and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-hydroxyquinoline N-oxides.** *Biochem J* 1956, **63**(1):130-137.

43. Novick RP: **Autoinduction and signal transduction in the regulation of staphylococcal virulence.** *Mol Microbiol* 2003, **48**(6):1429-1449.

44. Deziel E, Lepine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG: **Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication.** *Proc Natl Acad Sci U S A* 2004, **101**(5):1339-1344.

45. Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C: **Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*.** *J Bacteriol* 2002, **184**(23):6472-6480.

46. Lepine F, Milot S, Deziel E, He J, Rahme LG: **Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas aeruginosa*.** *J Am Soc Mass Spectrom* 2004, **15**(6):862-869.

47. Machan ZA, Taylor GW, Pitt TL, Cole PJ, Wilson R: **2-Heptyl-4-hydroxyquinoline N-oxide, an antistaphylococcal agent produced by *Pseudomonas aeruginosa*.** *J Antimicrob Chemother* 1992, **30**(5):615-623.

48. Bronner S, Monteil H, Prevost G: **Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications.** *FEMS Microbiol Rev* 2004, **28**(2):183-200.

49. Karlsson-Kanth A, Tegmark-Wisell K, Arvidson S, Oscarsson J: **Natural human isolates of *Staphylococcus aureus* selected for high production of proteases and alpha-hemolysin are σ^B deficient.** *Int J Med Microbiol* 2006, **296**(4-5):229-236.

50. Shopsin B, Drlica-Wagner A, Mathema B, Adhikari RP, Kreiswirth BN, Novick RP: **Prevalence of *agr* dysfunction among colonizing *Staphylococcus aureus* strains.** *J Infect Dis* 2008, **198**(8):1171-1174.

51. Sugiyama Y, Okii K, Murakami Y, Yokoyama T, Takesue Y, Ohge H, Sueda T, Hiyama E: **Changes in the *agr* locus affect enteritis caused by methicillin-resistant *Staphylococcus aureus*.** *J Clin Microbiol* 2009, **47**(5):1528-1535.

52. Traber KE, Lee E, Benson S, Corrigan R, Cantera M, Shopsin B, Novick RP: ***agr* function in clinical *Staphylococcus aureus* isolates**. *Microbiology* 2008, **154**(Pt 8):2265-2274.

53. Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR, Bohach GA: **Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells**. *Infect Immun* 1999, **67**(9):4673-4678.

54. Vaudaux P, Francois P, Bisognano C, Kelley WL, Lew DP, Schrenzel J, Proctor RA, McNamara PJ, Peters G, Von Eiff C: **Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes**. *Infect Immun* 2002, **70**(10):5428-5437.

55. Vann JM, Proctor RA: **Cytotoxic effects of ingested *Staphylococcus aureus* on bovine endothelial cells: role of *S. aureus* alpha-hemolysin**. *Microb Pathog* 1988, **4**(6):443-453.

56. D'Argenio DA, Calfee MW, Rainey PB, Pesci EC: **Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants**. *J Bacteriol* 2002, **184**(23):6481-6489.

57. Gotschlich A, Huber B, Geisenberger O, Togl A, Steidle A, Riedel K, Hill P, Tummler B, Vandamme P, Middleton B *et al*: **Synthesis of multiple N-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex**. *Syst Appl Microbiol* 2001, **24**(1):1-14.

58. Vial L, Lepine F, Milot S, Groleau MC, Dekimpe V, Woods DE, Deziel E: ***Burkholderia pseudomallei*, *B. thailandensis*, and *B. ambifaria* produce 4-hydroxy-2-alkylquinoline analogues with a methyl group at the 3 position that is required for quorum-sensing regulation**. *J Bacteriol* 2008, **190**(15):5339-5352.

59. Davies D: **Understanding biofilm resistance to antibacterial agents**. *Nat Rev Drug Discov* 2003, **2**(2):114-122.

60. Galli J, Ardito F, Calo L, Mancinelli L, Imperiali M, Parrilla C, Picciotti PM, Fadda G: **Recurrent upper airway infections and bacterial biofilms**. *J Laryngol Otol* 2007, **121**(4):341-344.
61. Holloway BW: **Genetics of Pseudomonas**. *Bacteriol Rev* 1969, **33**(3):419-443.
62. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM: **Common virulence factors for bacterial pathogenicity in plants and animals**. *Science* 1995, **268**(5219):1899-1902.
63. Sabat A, Krzyszton-Russjan J, Strzalka W, Filipek R, Kosowska K, Hryniewicz W, Travis J, Potempa J: **New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates.** *J Clin Microbiol* 2003, **41**(4):1801-1804.
64. Massey RC, Buckling A, Peacock SJ: **Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus***. *Curr Biol* 2001, **11**(22):1810-1814.
65. Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG: **Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus***. *Int J Med Microbiol* 2003, **293**(6):427-435.
66. Clinical and Laboratory Standards Institute (CLSI). **Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved Standard.** 2006.
67. Besier S, Smaczny C, von Mallinckrodt C, Krahl A, Ackermann H, Brade V, Wichelhaus TA: **Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in cystic fibrosis lung disease**. *J Clin Microbiol* 2007, **45**(1):168-172.
68. Zaborina O, Lepine F, Xiao G, Valuckaite V, Chen Y, Li T, Ciancio M, Zaborin A, Petrof EO, Turner JR *et al*: **Dynorphin activates quorum sensing quinolone signaling in *Pseudomonas aeruginosa***. *PLoS Pathog* 2007, **3**(3):e35.

CHAPITRE 4

Infection of polarized airway epithelial cells by normal and small-colony variant strains of *Staphylococcus aureus* is increased in cells with abnormal cystic fibrosis transmembrane conductance regulator function and is influenced by NF-κB

4.1. Introduction de l'article

Le but de cette étude a été de développer et de caractériser un modèle d'infection à *S. aureus* permettant la comparaison de cellules épithéliales non-FK et FK ainsi que de vérifier l'hypothèse que les SCVs de *S. aureus* ont une habileté plus grande que les souches normales à infecter ces lignées cellulaires. Nous avons aussi étudié l'effet de l'interaction entre le CFTR et NF-κB sur le niveau d'infection de cellules épithéliales pulmonaires polarisées par des souches normales et SCVs. J'ai fait l'apprentissage de la méthode de culture au laboratoire du Dr André M. Cantin sous la supervision de la professionnelle de recherche Ginette Bilodeau. M. Gilles Grondin a fait les expériences de microscopie. J'ai mis au point le modèle d'infection et effectué l'ensemble des essais d'infection, des expériences de microbiologie et des PCR quantitatives en temps réel. J'ai rédigé l'ébauche de l'article, effectué la conception des figures et participé à la correction du manuscrit jusqu'à sa publication.

Référence de l'article :

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4.2. Résumé en français de l'article

L'infection de cellules non phagocytaires par *Staphylococcus aureus*, et plus particulièrement par les SCVs, pourrait contribuer à la persistance de ce pathogène dans les voies pulmonaires des patients FK. On suppose également que le développement d'infections chroniques est facilité par l'état proinflammatoire des voies respiratoires FK induit par une activation anormale de NF-κB. Le but de cette étude était de comparer l'infection de cellules épithéliales non-FK et FK par des souches de *S. aureus* (normal et SCVs) ainsi que de déterminer l'impact de l'interaction entre le CFTR et NF-κB sur le niveau d'infection de ces cellules. Nous avons développé un modèle d'infection à *S. aureus* utilisant des cellules épithéliales pulmonaires polarisées cultivées à l'interface air-liquide et exprimant de petits ARN en tête d'épingle (shRNA) dirigés contre le CFTR afin de mimer la condition FK. Une paire de co-isolats FK génétiquement liés avec le phénotype normal et SCV a été caractérisée et utilisée. L'infection des deux lignées cellulaires (non-FK et FK) était plus prononcée avec la souche SCV qu'avec sa contrepartie normale. Cependant, les souches normales et SCVs infectaient plus les cellules FK que les cellules non-FK. De plus, l'inhibition de la fonction CFTR par le CFTR*inh*-172 augmentait le niveau d'infection à *S. aureus*. L'activation expérimentale de NF-κB augmentait aussi le niveau d'infection des cellules épithéliales pulmonaires polarisées par *S. aureus*, ce qui pourrait être associé avec les résultats obtenus lorsque la fonction du CFTR était inhibée ou compromise. Cette étude supporte l'hypothèse que l'état proinflammatoire des tissus FK facilite l'infection des cellules épithéliales pulmonaires par *S. aureus*.

4.3. L'article scientifique

Infection of polarized airway epithelial cells by normal and small-colony variant strains of *Staphylococcus aureus* is increased in cells with abnormal CFTR function and is influenced by NF-κB

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Running title: CFTR function protects against *S. aureus* infection

Keywords: chronic infections, cellular invasion, LPS, TNF-α, cystic fibrosis

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ABSTRACT

The infection of non-phagocytic host cells by *Staphylococcus aureus* and more particularly by small-colony variants (SCVs) may contribute to the persistence of this pathogen in the lungs of cystic fibrosis (CF) patients. The development of chronic infections is also thought to be facilitated by the proinflammatory status of CF airways induced by an activation of NF-κB. The aim of this study was to compare the infection of non-CF and CF-like airway epithelial cells by *S. aureus* strains (normal and SCVs), and to determine the impact of the interaction between CFTR and NF-κB on the infection level of these cells by *S. aureus*. We developed an *S. aureus* infection model using polarized airway epithelial cells grown at the air-liquid interface and expressing short hairpin RNAs directed against CFTR to mimic the CF condition. A pair of genetically related CF co-isolates with the normal and SCV phenotypes was characterized and used. Infection of both cell lines (non-CF and CF-like) was more productive with the SCV strain than that observed with its normal counterpart. However, both normal and SCV strains infected more CF-like than non-CF cells. Accordingly, inhibition of CFTR function by CFTR*inh*-172 increased *S. aureus* infection. Experimental activation of NF-κB also increased the infection level of polarized pulmonary epithelial cells by *S. aureus*, an event that could be associated with that observed when CFTR function is inhibited or impaired. This study supports the hypothesis that the proinflammatory status of CF tissues facilitates the infection of pulmonary epithelial cells by *S. aureus*.

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal single gene disease affecting Caucasians. This disorder is autosomal recessive and is caused by mutations affecting the function of the Cystic Fibrosis Conductance Regulator (CFTR), which is a cyclic 5'-monophosphate (cAMP)-regulated chloride channel localized in the apical membrane of epithelial cells. The malfunction of CFTR has an impact on multiple systems and mainly affects the respiratory and the gastrointestinal tracts. Ultimately, the majority of patients with CF will succumb from respiratory failure subsequent to chronic bacterial infections (31, 48). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are currently the most common pulmonary pathogens in North American subjects with CF (7, 13).

S. aureus is an important human pathogen which has the ability to cause both life-threatening diseases as well as chronic and difficult-to-treat infections of several organs and tissues (2, 20, 45). One of the mechanisms thought to be involved in the development of chronic *S. aureus* infections is the ability to persist within non-phagocytic host cells, which confers to the bacterium protection against the immune system and the action of antibiotics (1, 6). It was indeed observed that antibiotic treatments are often ineffective against *S. aureus* infecting CF lungs and are subsequently often associated with relapsing infections (19, 26). These relapsing infections are thought to re-emerge from bacteria persisting inside host cells (30). Jarry and Cheung (23) suggested that the fate of intracellular *S. aureus* may actually differ in CF epithelial cells compared to that seen in non-CF cells. Consequently, the ability of *S. aureus* to persist within epithelial cells may represent an important factor contributing to the specific persistence of *S. aureus* in CF patients.

How CFTR malfunction promotes pulmonary infections is still not completely understood. It was argued that bacterial lung colonization may be facilitated by the proinflammatory status of CF tissues and it was shown that dysfunctional CFTR is associated with an elevated level of NF-κB mediated IL-8 signalling in the airways (25, 49, 56, 58). Recent studies convincingly demonstrated that CFTR is indeed a negative regulator of NF-κB mediated innate immune response (22, 64). Interestingly, it is now known that *S. aureus* can activate NF-κB in airway epithelial cells (47) and that NF-κB may influenced the interaction between this bacteria and host cells (41, 66). It is thus possible that the proinflammatory status induced through NF-κB activity in CF airways encourages *S. aureus* intracellular infections and, consequently, the development of chronic infections.

Small-colony variants (SCVs) are bacteria often isolated from chronic infections as in the case of lung infections in CF patients but also in infections such as osteomyelitis, septic arthritis, infection of orthopaedic devices and bovine mastitis (1, 38, 45). SCVs are characterized by either a dysfunctional oxidative metabolism or a lack in thymidine biosynthesis, both causing an alteration in the expression of virulence factors, a slower growth and a loss of colony pigmentation (45). Whereas normal strains usually repress cell-surface proteins and express exoproteins when growing toward the stationary phase (40), SCVs stably express SigB-dependent genes encoding cell-surface proteins such as adhesins instead of activating the quorum-sensing dependent *agr* system and producing exoproteins (38). These differences in expression of virulence factors result in an increased ability to adhere to host components (36) and to form biofilm (34, 37) and should also be linked to the ability of SCVs to invade and persist within host cells (45).

The aims of this study was to develop and characterize a *S. aureus* infection model allowing the comparison of non-CF and CF-like polarized pulmonary epithelial cells as well as to verify the assumption that SCVs have a greater ability to infect these cell lines compared to normal

S. aureus. We also studied the impact of the interaction between CFTR and NF-κB on the infection level of polarized pulmonary epithelial cells by normal and SCV strains.

MATERIALS AND METHODS

Bacterial strains

The strains CF07-L, CF07-S and CF1A-L were previously described (34, 36-38). Briefly, CF07-L and CF07-S are genetically related *S. aureus* strains co-isolated from a CF patient and respectively have a normal and a SCV phenotype (37, 38). The genetic relatedness of these two strains was previously demonstrated by multiple-locus variable-number of tandem repeat analysis (MLVA) (37). Multilocus sequence typing (MLST) was additionally performed here using the procedure previously described (16). Sequences for internal fragments of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* housekeeping genes were obtained for CF07-L and CF07-S strains and submitted to the MLST homepage (http://www.mlst.net) in order to determine their sequence type (ST). CF1A-L was isolated from a CF patient and has a normal growth phenotype (37).

Growth curves

Brain heart infusion (BHI) cultures inoculated at an $A_{600\ nm}$ of 0.1 were incubated at 35°C/225 RPM. Samples were taken at different points in time for CFU determination by spreading 10-fold dilutions on trypticase soy agar (TSA) plates (BD, Mississauga, ON, Canada). TSA plates were incubated at 35°C for 24 and 48 h before enumeration of colonies for CF07-L and CF07-S, respectively.

Hemolysis

Bacterial suspensions (0.5 McFarland) prepared for each strain were spotted (2 μl) on Mueller Hinton agar (BD) supplement with 5% horse blood (Oxoid, Nepean, ON, Canada). Hemolysis was observed after 24 h of incubation at 35°C.

Quantitative PCR

BHI broth cultures ($A_{600\ nm}$ of 0.1) were grown for 3 h at 35°C/225 RPM. RNA extraction, cDNA synthesis and qPCR for the evaluation of *asp23*, *fnbA*, *hld* and *hla* expression were performed as previously described (34, 36-38). The relative expression ratios were calculated by using the cycle threshold (C_t) of the housekeeping gene *gyrB* (*n*-fold expression = $2^{-\Delta Ct}$, where ΔC_t represents the difference between the C_t of the gene studied and the C_t of *gyrB* for each strain).

Cell lines and growth conditions

The human airway epithelial cell line Calu-3 (ATCC HTB 55) and its derivatives were cultured in Eagle's minimum essential medium supplemented with 0.1mM of minimum essential medium nonessential amino acids, 1 mM of sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml of Fungizone and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. In order to compare non-CF and CF-like cells in the Calu-3 background, we used the stable cell lines expressing *shCFTR*$_{ALTER}$ and *shCFTR*, respectively (42). shCFTR$_{ALTER}$ cells act as the control that express a short hairpin RNA (shRNA) that does not match nucleotide sequences from any known cloned cellular proteins whereas shCFTR cells express a shRNA directed against CFTR. For routine culture of shCFTR$_{ALTER}$ and shCFTR cell lines, 4 μg/ml of puromycin was added to culture media. All cell culture reagents were purchased from Wisent (St-Bruno, QC, Canada).

Transepithelial electrical resistance measurements

Transepithelial electrical resistance (TEER) measurements were done by using chopstick electrodes and an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL, USA) on cells grown at the air-liquid interface in Transwells (Corning, NY, USA) as described by Grainger *et al.* (21). Before each measurement, medium was added to the apical chamber. TEER was calculated by subtracting the resistance of a cell-free culture insert and correcting for the surface area of the Transwell cell culture support.

cAMP-dependent iodide efflux assays

cAMP-dependent iodide efflux assays were performed as previously described (9, 10) in order to assess the functionality of CFTR. Briefly, cells were cultured to 90% confluence in six-well plates and labeled with 15 μCi/well ^{125}I for 1 h at room temperature. The cells were washed six times and covered with efflux buffer (119 mM Na gluconate, 1.2 mM K_2HPO_4, 0.6 mM KH_2PO_4, 25 mM $NaHCO_3$). Supernatants were collected over time, and the buffer was replaced with fresh efflux buffer every minute. When indicated, efflux buffer containing 0.5mM 2'O-dibutyryl cAMP, 10 μM 3-isobutyl-1 methylxanthine, and 10 μM forskolin (cAMP buffer) was added. Supernatants were then collected, and fresh cAMP buffer was added to cells every minute for the remaining of the experiment. The radioactivity of the supernatants was determined in a γ-counter. 2'O-dibutyryl cAMP, 3-isobutyl-1 methylxanthine, and forskolin were from Sigma (Oakville, ON, Canada).

Apical surface liquid measurements

Cells were grown for 9-10 days in Transwells at the air-interface. The basal medium was then supplemented or not with 0.5mM 2'O-dibutyryl cAMP, 10 μM 3-isobutyl-1 methylxanthine, and 10 μM forskolin, the cells were incubated at 37°C/5% CO_2 for 24 h and the volume of apical surface liquid was measured.

Cell infection assays

Cell infection assays were performed as previously describes with few modifications (35). Briefly, cells were seeded at ~1.5 x 10^5 cells/insert on 12-wells Transwells and cultured for 9 to 10 days in an air-liquid system. This yielded to 1 × 10^6 nucleus/insert independently of the cell line used (See protocol for Hoescht staining below). The complete medium in basal compartments was replaced by the invasion medium (1% FBS and no antibiotics) 18 h before the invasion assay. Inocula were prepared by suspending bacteria grown 20 h on BHI agar plates in ice-cold phosphate buffered saline (PBS). Bacteria were then washed three times in ice-cold PBS and suspended in the invasion medium supplemented with 0.5% BSA at a density of approximately 4 × 10^8 CFU/ml. Results were corrected according to inoculum density. Cells were washed twice with PBS and 250 µl of bacterial suspension were apically added to each insert. Invasion was allowed for 3 h, inserts were emptied and washed three times with PBS. Invasion medium supplemented with 20 µg/ml of lysostaphin (Sigma) was then added to both apical and basal compartments to kill extracellular bacteria and the cells were further incubated for the indicated time. The use of lysostaphin to kill extracellular normal and SCV bacteria was previously validated in cell invasion assays (38, 61, 63, 65, 67). The concentration of lysostaphin used in our assays was at least 2.5 fold higher than the minimal inhibitory concentrations of lysostaphin determined against a collection of 40 strains of *S. aureus* that included 17 normal-SCV co-isolate pairs. The MIC of lysostaphin against strain CF07-L and SCV CF07-S was identical (4 µg/ml). DMSO, CFTR*inh*-172 (EMD, Gibbstown, NJ, USA), human recombinant TNF-α (Bioshop, Burlington, ON, Canada), LPS from *Escherichia coli* 0127:B8 (Sigma) and the NF-κB activation inhibitor 6-amino-4-(4-phenoxy-phenylethylamino)quinazoline (QNZ) (EMD) were added after bacterial invasion when indicated in order to evaluate the post-invasion effect of these molecules. Twenty-four hours post-invasion and/or 1 h before cell lysis, cells were washed again once with PBS and the invasion medium supplemented with lysostaphin was replaced to ensure that no bacteria survive or replicate outside cells. At the end of the incubation time and following three washes with PBS, cells were detached with 100 µl of trypsin 0.25% and lysed for 10 min by the addition of 400 µl of water containing 0.05% of Triton X-100. The use of triton X-100

allowed optimal recovery of intracellular *S. aureus* bacteria. Lysates were serially diluted 10-fold and plated on agar for CFU determination.

Fluorescence microscopy

For microscopic examination following the cell invasion protocol, cells on inserts were fixed 120 min in 4% paraformaldehyde in PBS instead of being lysed. They were then permeabilized with a 5 min incubation in 50% methanol at -20°C followed by a 10 min incubation at room temperature in PBS supplemented with 50 mM glycine, 0.06% saponin, 0.06% Tween 20, 0.5% NP40 and 0.5% Triton X-100 (PBSP). The permeabilized cells were then incubated 30 min with image-iT® FX signal enhancer (Invitrogen, Burlington, ON, Canada). *S. aureus* was probed with the rabbit antibody AB20920 (Abcam, Cambridge, MA, USA) 1:250 and F actin was stained with AlexaFluor 488 Phalloidin (Invitrogen) 1: 20 in PBS with 2% normal goat serum, 2% BSA and 0.45 % of fish gelatin (PBSB) for 180 min at room temperature. After five washes in PBS with 0.01% saponin and 0.01% tween-20 (PBSD), primary antibodies were detected by an incubation of 90 min at room temperature with AlexaFluor 555 goat anti-rabbit IgG (Invitrogen) at a dilution of 1:1000 in PBSB. The DNA was then stained 10 min with Hoechst (Invitrogen) at 1:5000 after five washes in PBSD and five washes in water. Inserts were washed five other times in water and were mounted in Prolong Gold anti-fade reagent (Invitrogen). Pictures were taken using an inverted Olympus IX 70 microscope with a Cool SNAP-Pro *cf.* monochrome camera and Image-Pro Plus software or an Olympus Fluoview FV 300 confocal system or a Zeiss microscope equipped with the Apotome system. To obtain transversal sections images, fixed inserts were embedded in paraffin with standard method and 7μm cross sections were deparaffinised before labeling.

Light and transmission electron microscopy

Cells on inserts were fixed for 1 h in 0.1 M cacodylate buffer (pH 7.2) containing 2% paraformaldehyde, 2.5% glutaraldehyde, 2 mg/ml tannic acid, 0.3 mg/ml saponin, 50 mM

KCl and 5 mM $MgCl_2$, rinsed three times in 0.1 M cacodylate buffer and post-fixed for 1 h in dark with OsO_4 at 4°C. Samples were then washed three times in H_2O, incubated 1 h in a solution of 2 % uranyl acetate and washed again three times in H_2O. Samples were dehydrated by subsequent 5 min incubations in ethanol 50%, 70%, 90%, 100% (3x) and 1:1 ethanol:acetone (2x). Samples were incubated in a solution of lead acetate, washed twice in 1:1 ethanol:acetone, washed twice in propylene oxide and embedded in Epon. For light microscopy, sections of 1 µm were mounted on slides and stained in toluidine blue (1.0% wt/vol toluidine blue in 1 % aqueous sodium borate) for 1 min and examined in a Zeiss Axio Imager.Z1 light microscope. For electron microscopy, sections of 90 nm were examined in a Philips EM 201 electron microscope at an operating voltage of 60Kv.

Cell viability assays

The effect of CFTR*inh*-172 on cell viability was determined by a XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide)-based assay. Briefly, Calu-3 cells were seeded at ~2 x 10^4 cells/well in 96-well plates and grown to confluence. The cells were then incubated with CFTR*inh*-172 at concentrations up to 300 µM for 24 h at 37°C and 5% CO_2 in complete medium. Cells were washed 3 times in Earle's balanced salt solution (EBSS) (Invitrogen) and incubated with 100 µl/well of a PBS-EBSS 1:1 mixture containing 3.75 µg/ml of phenazine methosulfate (Sigma) and 0.5 mg/ml of the XTT reagent (Invitrogen) for 45 min in the dark at room temperature. The $A_{450\ nm}$ was measured using a microplate reader (Bio-Tek Instruments). All assays were performed using four replicates.

Statistical analysis

Intracellular bacterial CFU ratios (see Figure legend for specifications) were transformed in base-10 logarithm values before being used for statistical analyses that were carried out with the GraphPad Prism Software (v.5.00). Statistical analyses on qPCR data were done on mean

ΔC_t. Statistical tests used for the analysis of each experiment are specified in the figure legends.

RESULTS

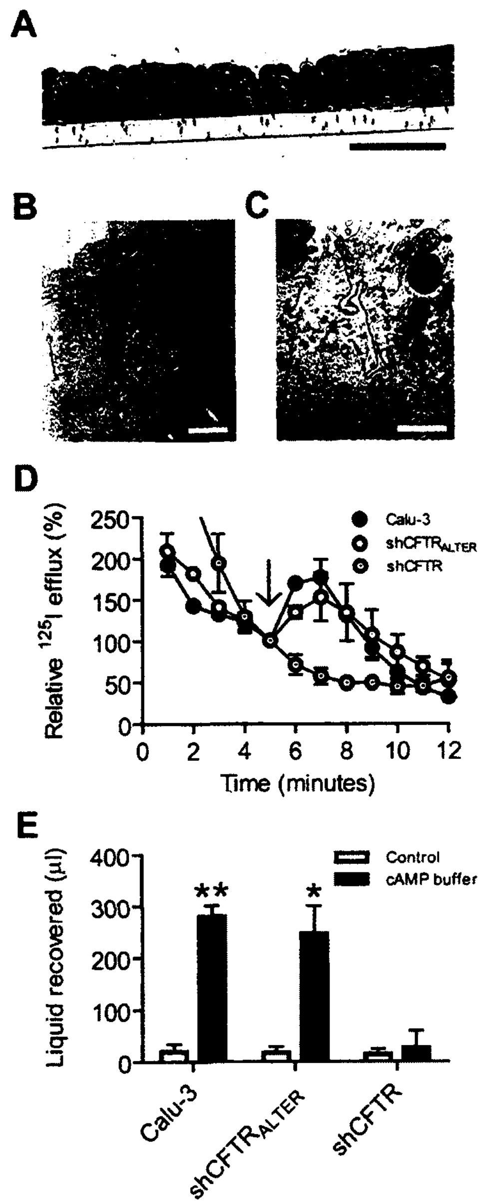


FIGURE 1. Characterization of an infection model allowing the comparison of non-CF and CF-like polarized airway epithelial cells grown at the air-liquid interface. (A) Optical microscopy showing the transversal cross section of polarized Calu-3 cells grown for 10 days at the air-liquid interface. Cells were stained with toluidine blue. Scale bar is 50 μm. (B) Electron microscopy demonstrating that Calu-3 cells grown for 10 days at the air-liquid interface are polarized according to the specialization of their apical and basolateral sides. Scale bar is 5 μm. (C) Electron microscopy confirming that intimate contacts are formed between Calu-3 cells. Scale bar is 1 μm. cAMP-dependent iodide efflux assays (D) and apical surface liquid measurements (E) confirming the functionality of CFTR in the Calu-3 and $shCFTR_{ALTER}$ control cell lines and demonstrating the abnormal CFTR function in shCFTR cells. Significant differences between the volume of liquid recovered from control and cAMP-stimulated cells are indicated for each cell lines (*, $P < 0.05$; **, $P < 0.01$; unpaired *t*-test). In (D) the efflux curves are expressed as percentages of the control measured before the addition of the cAMP buffer (indicated by an arrow). Data are presented as means with standard deviations from at least two independent experiments.

Development of an infection model using non-CF and CF-like polarized airway epithelial cells grown at the air-liquid interface. The Calu-3 cell line produces features of differentiated human airway epithelial cells and forms tight junctions when grow *in vitro*, which allow it to be used as a model of airway epithelial barrier (21). As shown by Fig. 1A, the Calu-3 cells form a pseudo-stratified layer of columnar cells with a rugged apical topography when grown at the air-liquid interface for 10 days. Apical microvilli and tight junctions were also detected by transmission electron microscopy (Fig. 1B and 1C). After few days of culture, Calu-3 cell layers produced an effective barrier to hydrostatically driven medium from the basolateral to the apical side and reached a transepithelial electrical resistance above 1000 $\Omega \cdot cm^2$ (data not shown).

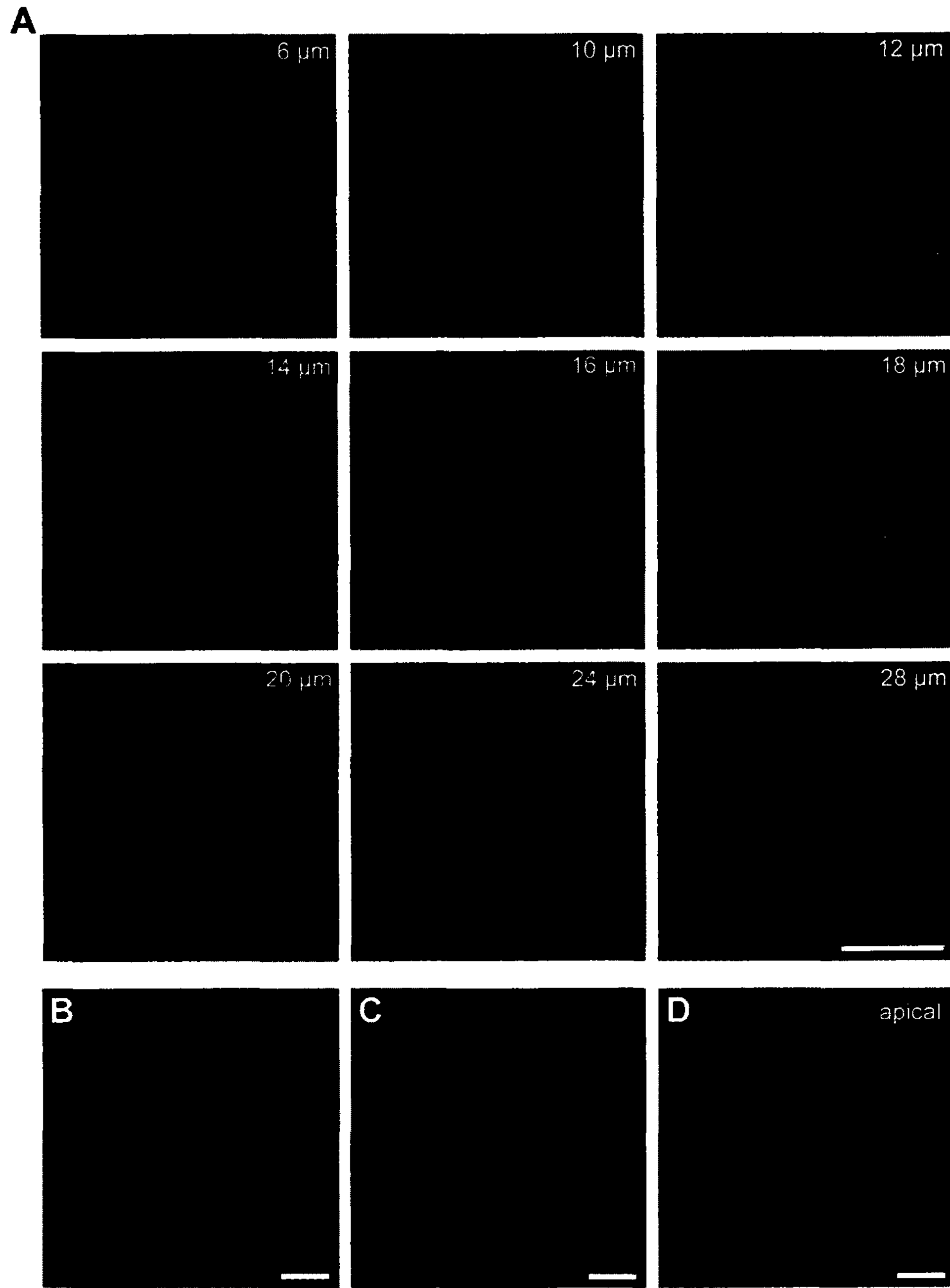


FIGURE 2. Confocal microscopy of non-CF and CF-like polarized airway epithelial cells infected by *S. aureus*. (A) Z-series images of Calu-3 cells infected with CF07-L 24 h post-invasion are shown. Distances from the bottom of the insert are indicated in microns (µm) for each image. Scale bar is 50 µm. Stacked images of selected layers obtained with confocal microscopy showing CF07-L (B) or CF07-S (C) *S. aureus* bacteria within shCFTR cells. Scale

bar is 5μm. (D) Transversal section of $shCFTR_{ALTER}$ cells grown on Transwells infected with the SCV CF07-S. Scale bar is 10 μm. *S. aureus* bacteria, nucleus (only in D) and F-actin are stained red, blue and green, respectively.

In order to compare non-CF and CF-like cells in the Calu-3 background, stable cell lines expressing *shCFTR*$_{ALTER}$ and *shCFTR* were used (42). As mentioned above, $shCFTR_{ALTER}$ cells act as the control that express a short hairpin RNA (shRNA) that does not match nucleotide sequences from any known cloned cellular proteins whereas shCFTR cells express a shRNA directed against CFTR. Fig. 1D and 1E confirm that shCFTR cells have an abolished CFTR activity while the functionality of CFTR is preserved in $shCFTR_{ALTER}$ cells. We first performed cAMP-dependent iodide efflux assays in order to assess the functionality of CFTR (9, 10). Calu-3 and $shCFTR_{ALTER}$ cells previously loaded with ^{125}I and stimulated with IBMX, forskolin and dibutyryl cAMP secreted high level of ^{125}I whereas this was not observed with the shCFTR cells (Fig. 1D). This confirmed the abnormal CFTR activity in shCFTR cells. The apical-basolateral fluid transport across cultured epithelial cells is also associated with the activity of CFTR (17). Fig. 1E shows that the transport of liquid across the epithelial barrier formed by shCFTR cells was indeed not stimulated with IBMX, forskolin and dibutyryl cAMP in comparison to Calu-3 and $shCFTR_{ALTER}$ cells. This further demonstrated that the normal level of CFTR activity is importantly reduced by the expression of *shCFTR*. Noteworthy, all cell lines still reached a significant transepithelial electrical resistance when grown at the air-liquid interface and no significant difference in the number of Hoescht-stained nucleus per microscopic field was observed between cell lines after 10 days of growth in these conditions (data not show). These Calu-3 derived cell lines were used in order to develop an infection model of polarized airway epithelial cells allowing the comparison of non-CF and CF-like backgrounds.

To allow comparison of outcomes when using different bacterial strains, the cell invasion protocol was adjusted to set the internalization level of normal strains (*e.g.* CF07-L) below saturation (<1CFU/cell) but above the CFU detection limit of the system. In order to confirm the internalization of bacteria in polarized epithelial cells, we acquired z-series images of Calu-3, $shCFTR_{ALTER}$ and shCFTR cells infected with a normal *S. aureus* (CF07-L) strain and its SCV counterpart (CF07-S) using confocal microscopy at 24 and 48 h post-invasion. Bacteria and cellular F actin were detected using a specific *S. aureus* antibody and phalloidin, respectively (See Material and Methods). Detection of actin was used to reveal cell shapes as actin is known to have extensive and intimate interactions with cellular membranes. Fig. 2A shows typical z-series images obtained with Calu-3 cells infected with *S. aureus* 24 h post-invasion. The presence of intracellular bacteria validated our infection protocol. Intracellular bacteria were also detected within $shCFTR_{ALTER}$ and shCFTR cells infected with either CF07-L or CF07-S 24 h post-invasion, but also 48 h post-invasion (data not shown). Fig 2B and 2C further supports the validity of the use of the anti-*S. aureus* antibodies in the detection of intracellular bacteria by clearly showing cocci inside shCFTR cells infected with CF07-L or CF07-S, respectively. The different morphologies of cells shown in Fig 2B and 2C are explained by the z-postion of the confocal microscopy section within the pseudo-stratified cell layer, which typically presents cells with various sizes and shapes. The cell section showed in Fig 2B is on top of the cell layer (stacked microscopic images taken from 20 to 23 μm from the bottom of the insert) whereas the cell section in Fig 2C is in the middle of the cell layer (stacked microscopic images taken from 7.5 to 10 μm from the bottom of the insert). Fig. 2D shows a transversal section of $shCFTR_{ALTER}$ cells grown on Transwells infected with the SCV CF07-S where bacteria, actin and nucleus (stained in blue using Hoescht staining) are revealed. Images from Fig. 2 clearly show the intracellular localization of *S. aureus* when polarized epithelial cells are infected according to our protocol. Overall, this section validates an intracellular infection model using non-CF and CF-like polarized airway epithelial cells derived from the Calu-3 cell line.

Characterization of the genetically related normal CF07-L and SCV CF07-S strains co-isolated from a CF patients. The genetic relatedness of the CF07-L and CF07-S co-isolates was demonstrated by MLVA (37) with a protocol previously described and validated (33, 50, 57). Accordingly, the sequences of MLST-gene fragments we determined for strains CF07-L and CF07-S were 100% identical and corresponded to the ST45 lineage, which is frequent among human isolates (12). Some of our previous studies examined the growth, adhesion properties, biofilm formation and virulence gene expression of the SCV strain CF07-S (34, 36-38), but these characteristics were not always directly compared to those of the normal phenotype counterpart CF07-L. Fig. 3 now compares the growth characteristics of CF07-L and CF07-S strains and the expression of some key virulence genes. Fig. 3A and 3B confirmed that the growth rate of CF07-L is higher than that of CF07-S and that CF07-S forms pin-point colonies when streaked and grown on agar plates. Noteworthy, CF07-S is an SCV auxotroph for menadione, *i.e.*, it grows at a normal rate when in presence of this supplement (38). The SCV phenotype of CF07-S is relatively stable and reversion to the normal phenotype is rarely observed when the strain is streaked on agar plates (Fig. 3C). Accordingly, non-SCV colonies were rarely detected from CF07-S inocula used for cell invasion assays (*e.g.* detection threshold of approximately 2.5×10^{-7} normal/SCV colonies). The low reversion rate of CF07-S is thus very useful for our cell invasion assays that compare the intracellular persistence of normal and SCV phenotypes. The use of a naturally occurring SCV instead of a laboratory-derived mutant (*e.g.* a SCV *hemB* mutant) that possibly lacks compensatory mutations and the intrinsic ability of natural SCV to switch back to the normal phenotype further support the choice of the CF07-S and CF07-L co-isolate pair for our experimental objectives. Indeed, the switch between the SCV and normal phenotypes and vice versa is thought to be an important part of *S. aureus* pathogenesis (61) and the use of naturally occurring SCVs rather than laboratory mutants should be encouraged in studies comparing *S. aureus* interactions with the host or host cells.

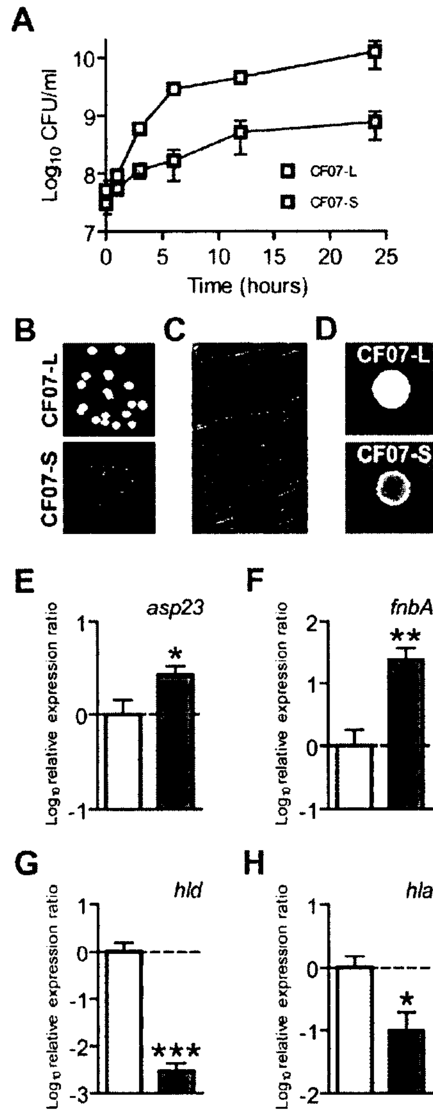
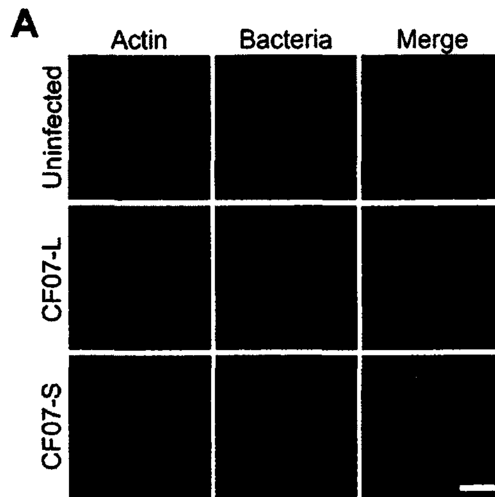
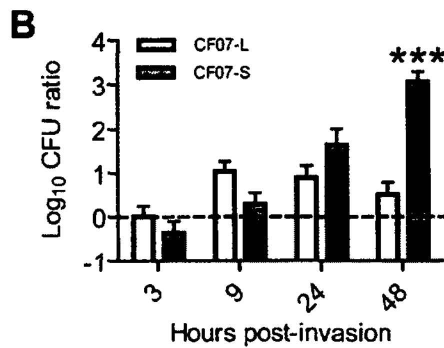


FIGURE 3. Characterization of the genetically related normal CF07-L and SCV CF07-S strains co-isolated from a CF patient. (A) Growth curves of CF07-L and CF07-S. (B) Colonies of CF07-L and CF07-S grown for 16 h on BHI agar plates at 35°C. (C) Streaks of CF07-S grown for 16h on a BHI agar plates at 35°C showing the stability of the SCV phenotype of the CF07-S strain. (D) Hemolytic activities of CF07-L and CF07-S. (E-H) Relative expression ratios for the genes *asp23*, *fnbA*, *hld* and *hla* in CF07-L and CF07-S. Results are normalized

according to CF07-L (dotted line) and are expressed as means with standard deviations. Significant differences between the expression of each gene in CF07-L and CF07-S are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired t-test).





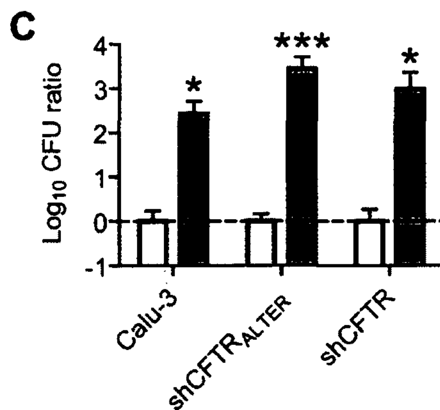


FIGURE 4. Both non-CF and CF-like polarized airway epithelial cells are more infected by a SCV than by a normal *S. aureus* strain. (A) Fluorescence microscopy showing that more SCV CF07-S bacteria are found in polarized cells 48 h post-invasion in comparison to the normal *S. aureus* strain CF07-L (here shown for the Calu-3 cells). *S. aureus* bacteria and cellular F-actin are stained red and green, respectively. Pictures are from stacked images covering the entire thickness of the cell layer obtained with a Zeiss microscope equipped with an ApoTome attachment. Scale bar is 50 μm. (B) Infection kinetics of Calu-3 cells by strains CF07-L and CF07-S. A significant difference between CFU ratios of strains CF07-L and CF07-S recovered from cells 48 h post-invasion is indicated (***, $P < 0.001$) and was determined by a two-way ANOVA with the Bonferonni's post test. Results are from 2 to 5 independent experiments and are normalized according to the inoculum ratio of CF07-L within cells 3 h post-invasion (note: the number of CFU recovered from CF07-L-infected cells 3 h post-invasion was 3.5±0.7 Log_{10} CFU/insert). (C) CFU ratios recovered from Calu-3, $shCFTR_{ALTER}$ and shCFTR cells infected with CF07-L and CF07-S 48h post-invasion. Significant differences between the CFU ratios of strains CF07-L and CF07-S are indicated for each cell line (*, $P < 0.05$; ***, $P < 0.001$; unpaired *t*-test). Results are normalized according to the inoculum ratio of CF07-L within each cell line (note: the numbers of CFU recovered from CF07-L-infected cells 48 h post-invasion were 4.5±0.7, 3.3±0.4 and 4.1±0.8 Log_{10} CFU/insert, for Calu-3, $shCFTR_{ALTER}$ and shCFTR cells, respectively). Data are from 2 to 3 independent experiments performed in duplicate and are presented as means with standard deviations.

The hemolytic activity of CF07-S was also lower than that of CF07-L (Fig. 3D) as usually observed for other SCV and normal strain pairs (45, 53). The expression of genes known to be differentially modulated between normal and SCV strains were also studied in CF07-L and CF07-S (Fig. 3E-H). Results confirmed that the SigB-activity marker gene *asp23* and the gene encoding the fibronectin binding protein A (*fnbA*) are both upregulated in CF07-S in comparison to that found in CF07-L (Fig. 3E-F). The expression of *asp23* and *fnbA* are indeed known to be positively influenced by the activity of SigB and to be upregulated in SCVs (34,

36-38, 54). As anticipated from previous studies (27, 34, 38), the *hld* (the effector of the *agr* system) and *hla* genes (encoding the α-hemolysin) were down-regulated in CF07-S in comparison to CF07-L (Fig. 3G-H). Overall, this section confirmed that strains CF07-L and CF07-S behave as usually observed for other *S. aureus* normal and SCV strain pairs (53).

Both non-CF and CF-like polarized airway epithelial cells are more infected by a SCV than by a normal *S. aureus* strain. SCVs are believed to have an increased ability to invade and persist within non phagocytic host cells (45). One of our aims was to confirm that SCVs could infect cells of the Calu-3 background, including $shCFTR_{ALTER}$ and shCFTR. We used the genetically related normal CF07-L and SCV CF07-S strains to perform those experiments. Fig. 4A shows fluorescent microscopy pictures obtained from non infected Calu-3 cells or from cells that were infected by CF07-L or CF07-S. These 48-h post-invasion images clearly demonstrate that the SCV CF07-S causes a higher level of intracellular infection comparatively to the normal strain CF07-L. Similar results were obtained with shCFTR and $shCFTR_{ALTER}$ cells using fluorescent microscopy (data not shown). Results of Fig 4B confirms the microscopy results and demonstrated that the SCV CF07-S has the ability to replicate within Calu-3 cells whereas CF07-L does not, explaining the higher level of intracellular infection 48-h post-invasion when these cells are infected by CF07-S. More precisely, non-normalized results correspond to 3.5±0.7 and 3±2 log_{10}CFU for CF07-L, and 3.0±0.5 and 6.4±0.7 log_{10}CFU for CF07-S, at 3 and 48 h post-invasion, respectively. Fig 4C shows that significantly more SCV CF07-S than normal CF07-L CFU were indeed recovered from the three cell lines 48 h post-invasion. These observations suggest that the SCV CF07-S is able to cause a higher level of cellular infection in comparison to its normal counterpart independently of the cell line used in this study. These observations were also confirmed using another pair of co-isolated and genetically-related SCV and normal CF strains (data not shown). Noteworthy, despite the use of naturally occurring SCV strains in these cell invasion assays, normal size colonies were rarely recovered from cells 48 h post-invasion.

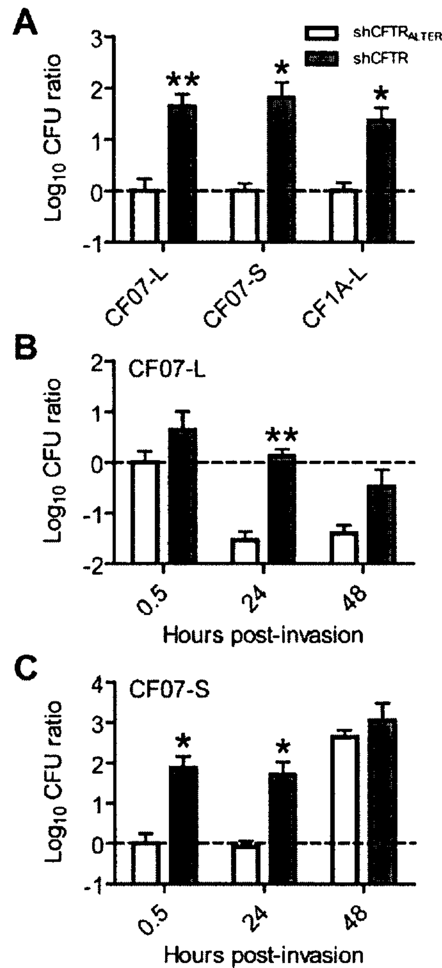


FIGURE 5. Normal and SCV strains infect CF-like cells more than non-CF cells. (A) CFU ratios recovered from $shCFTR_{ALTER}$ (non-CF) and shCFTR (CF-like) cells infected with CF07-L, CF07-S and CF1A-L, 24 h post-invasion. Significant differences between CFU ratios recovered from each cell line are indicated for each strain (*, $P < 0.05$; **, $P < 0.01$; unpaired *t*-test). Results are normalized according to the inoculum ratio of each strain within $shCFTR_{ALTER}$ cells (note: the numbers of CFU recovered from infected $shCFTR_{ALTER}$ cells 24 h post-invasion were 3.1±0.7, 4.0±0.6 and 3.3±0.4 Log_{10} CFU/insert, for CF07-L, CF07-S and CF1A-L, respectively). CFU ratios recovered from $shCFTR_{ALTER}$ and shCFTR cells 0.5, 24 and 48 h post-invasion for strains CF07-L (B) and CF07-S (C). Significant differences

between CFU ratios recovered from each cell line are indicated for each post-invasion time (*, $P < 0.05$; **, $P < 0.01$; two-way ANOVA with the Bonferonni's post test). Results are normalized according to the inoculum ratio of each strain within $shCFTR_{ALTER}$ cells 0.5 h post-invasion (note: the numbers of CFU recovered from infected $shCFTR_{ALTER}$ cells 0.5 h post-invasion were 4.5±0.4 and 3.9±0.8 Log_{10} CFU/insert, for 5B and 5C respectively). Data are from 2 to 4 independent experiments performed in duplicate and are presented as means with standard deviations.

Normal and SCV strains infect CF-like cells more than non-CF cells. Using non-polarized monolayers, it was previously suggested that the fate of *S. aureus* intracellular infections may be different in CF epithelial cells than in their normal counterpart (23). Consequently, we next compared infections of polarized non-CF and CF-like cell lines by normal *S. aureus* strains as well as by SCVs. Fig. 5A shows that more CFU from two normal and one SCV strains were recovered from shCFTR (CF-like) than from $shCFTR_{ALTER}$ (non-CF) cells 24 h post-invasion. This demonstrates that higher levels of infection can be obtained by *S. aureus* in polarized cells with an altered CFTR function compared to those reached in non-CF cells. Infection kinetics with CF07-L and CF07-S in both cell lines (non-CF and CF-like) were also done in order to discriminate between differences in infection levels caused by invasion (evaluated at 0.5 h post-invasion) or post-invasion mechanisms (evaluated at 24 and 48 h post-invasion). Fig. 5B shows that although there was not a significant difference between the invasion of CF07-L in both cells lines (0.5-h time point), the survival of the strain was higher in the CF-like shCFTR cells than that observed in the $shCFTR_{ALTER}$ cells 24 h post-invasion. On the other hand, the SCV CF07-S showed a significantly higher invasion rate in shCFTR cells in comparison to that found in $shCFTR_{ALTER}$ cells (Fig. 5C, 0.5-h time point), and thereafter, the high number of intracellular CFU recovered for the SCV strain was sustained up to 48 h post-invasion independently of the cell line used (Fig. 5C). Overall, these results show that polarized CF-like cells are more susceptible to infection by *S. aureus* strains (normal and SCV) than non-CF cells.

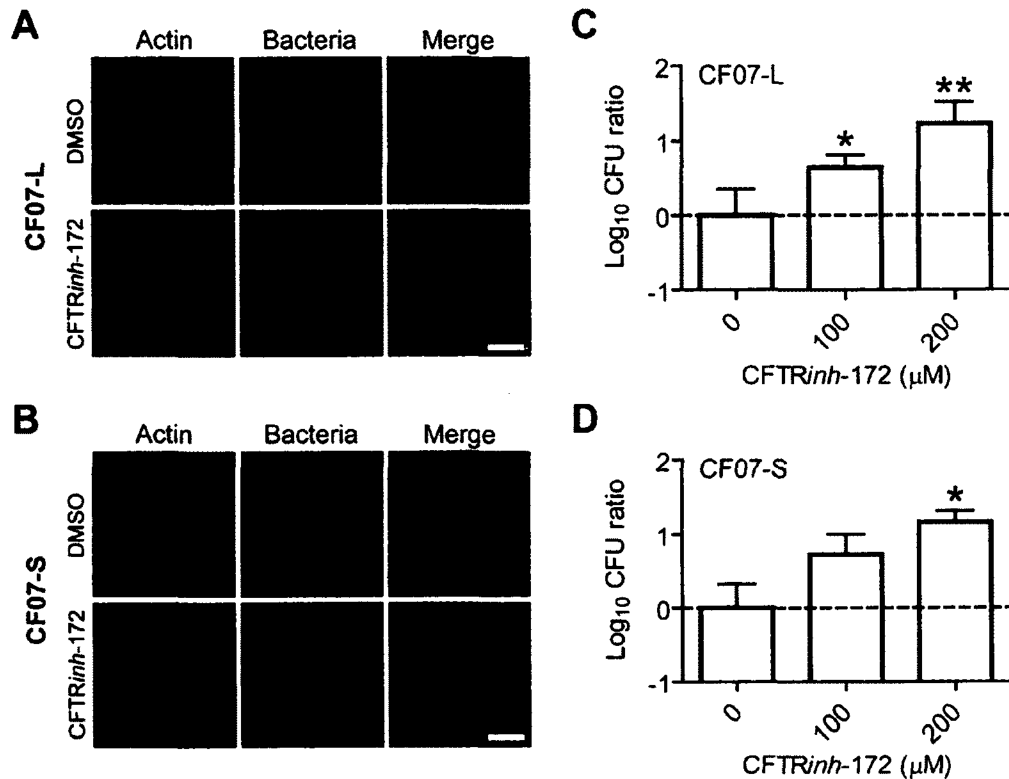


FIGURE 6. CFTR*inh*-172 confirms that a CFTR-dependent post-invasion mechanism helps reduce the infection of polarized cells by normal and SCV strains. Fluorescence microscopy of control (DMSO) and CFTR*inh*-172-treated Calu-3 cells infected with CF07-L (A) and CF07-S (B) 24 h post-invasion. *S. aureus* bacteria and cellular F-actin are stained red and green, respectively. Pictures are from stacked images covering the entire thickness of the cell layer obtained with a Zeiss microscope equipped with ApoTome attachment. Scale bar is 50 µm. CFU ratios recovered from Calu-3 cells infected with CF07-L (C) and CF07-S (D) 24 h post-invasion. Significant differences between CFU ratios recovered from DMSO- and CFTR*inh*-172-treated infected culture are shown (*, $P < 0.05$; **, $P < 0.01$; one-way ANOVA followed by the Dunnett's post test). Results are normalized according to the inoculum ratio of each strain within DMSO-treated cells (note: the numbers of CFU recovered from infected DMSO-treated cells 24 h post-invasion were 3.0±0.7 and 5.3±0.9 Log_{10} CFU/insert, for 6C and 6D respectively). Data are from 2 to 5 independent experiments performed in duplicate and are

presented as means with standard deviations. DMSO and CFTR*inh*-172 were added to cultures after bacterial invasion (*i.e.*, 0 h post-invasion).

CFTR*inh*-172 confirms that a CFTR-dependent post-invasion mechanism helps reduce the infection of polarized cells by normal and SCV strains. In order to further demonstrate the importance of CFTR function in the observed differences in infection levels between non-CF and CF-like cells, the CFTR inhibitor CFTR*inh*-172 (32) was used. As expected, we first showed that CFTR*inh*-172 inhibited the activity of CFTR in Calu-3 cells by using ^{125}I efflux assays and measurements of apical fluid transport under stimulation with IBMX, forskolin and dibutyryl cAMP (data not shown). Importantly, we also verified that incubation of cells with CFTR*inh*-172 at concentrations up to 300 μM did not significantly affect cell viability compared to untreated cells by using a XTT-based assay (one-way ANOVA followed by the Dunnett's post test). CFTR*inh*-172 was then added to cultures of polarized Calu-3 cells after allowing cell invasion (*i.e.*, 0 h post-invasion) in order to evaluate the post-invasion effect of CFTR activity on *S. aureus* infection levels. Fig. 6A shows that when CFTR*inh*-172 was added to cells infected with CF07-L, more bacteria were observed 24 h post-invasion. This experiment was also done with Calu-3 cells infected with the SCV CF07-S (Fig. 6B) and showed that the CFTR inhibitor further increases infection by this SCV. Fig. 6 also presents the CFU ratios recovered from Calu-3 cells treated with CFTR*inh*-172 (or control DMSO) and infected with CF07-L (Fig. 6C) and CF07-S (Fig. 6D) and reveals that our microscopic observations can be supported by statistically significant quantitative data. Overall, this section suggests that a CFTR-dependent post-invasion mechanism helps reduce the infection of polarized epithelial cells by both normal and SCV *S. aureus* strains.

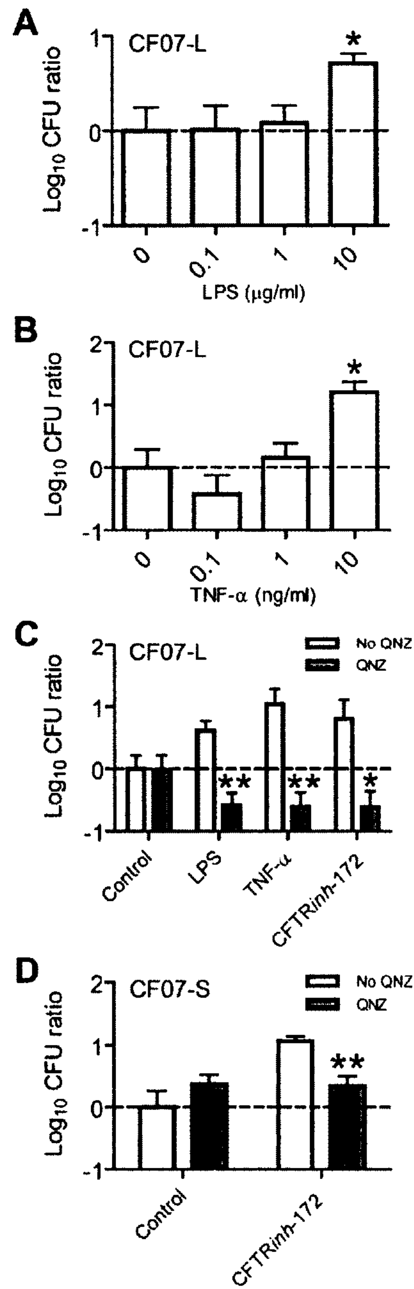


FIGURE 7. NF-κB activation increases the infection of pulmonary epithelial cells by *S. aureus*. CFU ratios recovered from Calu-3 cells infected with the normal *S. aureus* strain CF07-L in presence of different concentrations of LPS (A) and TNF-α (B). Significant

differences between CFU ratios recovered from control and treated cultures are shown (*, $P <$ 0.05; one-way ANOVA followed by the Dunnett's post test). Results are normalized according to the inoculum ratio of CF07-L within untreated cells (note: the numbers of CFU recovered from untreated CF07-L-infected cells 24 h post-invasion were 3.6±0.3 and 3.6±0.4 Log_{10} CFU/insert, for 7A and 7B respectively) and are from 3 independent experiments performed in duplicate. (C) Combined effects of the NF-κB activation inhibitor QNZ (100 nM) and LPS (10 μg/ml), TNF-α (10 ng/ml) and CFTR*inh*-172 (200 μM) on the infection of Calu-3 cells by CF07-L 24 h post-invasion. Significant differences between CFU ratios recovered from DMSO- and QNZ-treated cultures are shown (*, $P < 0.05$; **, $P < 0.01$; unpaired *t*-test). (D) Combined effects of QNZ and CFTR*inh*-172 on the infection of Calu-3 by the SCV strain CF07-S 24 h post-invasion. A significant difference between CFU ratios recovered from DMSO- and QNZ-treated cultures is shown (**, $P < 0.01$*; unpaired *t*-test). Results are normalized according to the inoculum ratio of each strain within untreated cells (note: the numbers of CFU recovered from untreated cells 24 h post-invasion were 3.9±0.5 and 5.7±0.3 Log_{10} CFU/insert, for 7C and 7D respectively). Data are from 2 to 5 independent experiments performed in duplicate and are presented as means with standard deviations (C and D). DMSO, TNF-α, LPS, CFTR*inh*-172 and QNZ were added to cultures after bacterial invasion (*i.e.*, 0 h post-invasion).

NF-κB activation increases the infection of pulmonary epithelial cells by *S. aureus*. We also tested the hypothesis that the increased infection level observed in CF-like cells or in Calu-3 cells treated with CFTR*inh*-172 is associated with an activation of NF-κB. Indeed, it was previously shown that CFTR is a negative regulator of NFκB-mediated innate immune response (22, 64) and that the NF-κB status may influence the interaction between *S. aureus* and the host cells (41, 66). Importantly, it was suggested that NF-κB may influence the internalization of *S. aureus* (41), but to date, there was no demonstration that NF-κB could influence the course of *S. aureus* infections after cellular invasion. Fig. 7A and 7B show that post-invasion treatments of cells with LPS and TNF-α, two well-known activators of NF-κB

(43), increased the CFU of CF07-L recovered from Calu-3 cells 24 h post-invasion. In order to be sure that these results were associated with NF-κB, we also used the NF-κB activation inhibitor QNZ (29, 59). Indeed, LPS- and TNF-α-induced *S. aureus* cellular infections were abolished in presence of the NF-κB activation inhibitor QNZ (Fig. 7C). Moreover, the presence of QNZ also abolished CFTR*inh*-172-induced *S. aureus* cellular infections with both normal (Fig. 7C) and SCV (Fig. 7D) strains. These results suggest that activation of NF-κB can increase the infection levels of pulmonary epithelial cells by both normal and SCV *S. aureus* strains after invasion and that inhibition of CFTR functions may trigger such a mechanism.

DISCUSSION

Several investigators have observed that the level of differentiation of cells used for bacterial invasion experiments may alter the interaction between pathogens and host cells (11, 18, 39, 44). We therefore determined that there was a need to develop and validate an infection model using CF polarized epithelial cells to study cellular invasion with bacterial pathogens commonly encountered by CF patients. Calu-3 cells can form a layer of polarized columnar cells with apical microvilli and tight junctions when grown at the air-liquid interface and such cells are thus useful as a model representative of the airway epithelium (21). This study developed and characterized a *S. aureus* infection model allowing the comparison of non-CF and CF-like backgrounds in polarized Calu-3 cells grown at the air-liquid interface. The use of Calu-3 cells expressing a short hairpin RNA directed against CFTR to create a CF-like background has been validated and discussed elsewhere (43).

Several studies suggested that SCVs have a greater ability to infect host cells (53, 60). Our results confirmed that this is true for both non-CF and CF-like polarized airway epithelial

cells. SCVs are indeed particularly endowed with strategies to infect host cells: their adherence and uptake by host cells are increased (36, 38, 62), they cause less damage to host cells (3, 4, 60) and seem to be more resistant to intracellular host defences (5, 52, 60). These abilities are related to the moderate virulence of SCVs that fail to activate the quorum-sensing dependent *agr* system and to the predominant role of the alternative sigma factor SigB that allows expression of genes encoding surface proteins, adhesins (such as the fibronectin-binding protein FnBA) and proteins involved in stress tolerance (34, 36-38).

One of the striking results of this study is that SCVs are more internalized by cells that have a defect in CFTR expression than by normal cells. The reason for this difference between both cell lines is not known, but it is possible that the SCV cellular receptor(s) may be differentially distributed or expressed in CF-like cells in comparison to non-CF cells. As an example, it was shown that CF cells express an increased number of asialylated glycolipids such as asialoGM1 (51), which is known to be a receptor for many pulmonary pathogens including *S. aureus* (28). We also cannot exclude that *S. aureus* invasion may be helped by an atypical distribution of the $\alpha_5\beta_1$ integrin (44) in shCFTR cells in comparison to that taking place in normal $shCFTR_{ALTER}$ cells. Indeed, *S. aureus* is able to invade host cells by indirectly binding to $\alpha_5\beta_1$ integrins via fibronectin (55), a strategy which may be especially important for SCVs that show a strong and sustained expression of *fnbA* (36, 62).

Our results strongly suggest that a defect in CFTR helps *S. aureus* to achieve higher levels of infection after invasion. Accordingly, we observed a significant reduction of infection with the SCV strain CF07-S by treating non-CF Calu-3 cells with activators of CFTR (IBMX, forskolin and dibutyryl cAMP) after cellular invasion (data not shown). Jarry and Cheung (23) suggested a model in which *S. aureus* escapes the normal endocytic/degradative pathway into the cytoplasm of CF host cells, thus avoiding degradation within the lysosomal compartment. Another study showed that CFTR regulates acidification and alters the bactericidal activity of

macrophages (14). It is thus possible that the antibacterial activity of CF epithelial cells is similarly altered and facilitates intracellular survival of *S. aureus*. Calu-3 cells are known to produce several antimicrobials (24, 46) and it is also possible that the production or activity of these molecules are differentially modulated in non-CF and CF-like backgrounds (15, 24). Moreover, it was observed that the induction of apoptosis is defective in CF cells in comparison to non-CF cells (8, 23, 56), and this may allow the population of bacteria to reach a higher density in CF cells before their ultimate death. The exact mechanism(s) for the very productive infections we observed in cells with abnormal CFTR functions remains unknown. While further investigations are required, such as studies using primary epithelial cell cultures from non-CF and CF patients, our results agree with the conclusion that normal CFTR functions help reducing S. *aureus* intracellular infection.

It was demonstrated that the internalization of *S. aureus* by bovine endothelial cells is associated with the status of NF-κB (41), but the impact of the activity of NF-κB on cellular mechanisms influencing *S. aureus* intracellular multiplication had never been investigated prior to the present study. It is well established that NF-κB regulates apoptosis (43) and it was recently observed that *S. aureus*-induced apoptosis is influenced by NF-κB (66). It is therefore possible that the induction of NF-κB delays apoptosis of infected host cells and allows *S. aureus* bacteria to reach a higher density per cell. This study shows that activation of NF-κB can indeed increase the infection levels of pulmonary epithelial cells by *S. aureus* 24h post-invasion and that inhibition of CFTR functions may trigger such a mechanism. However, the relation between CFTR, NF-κB and the induction of apoptosis during infection of airways epithelial cells still remain to be specifically studied.

This report supports the hypothesis that long-term colonization of lungs by *S. aureus* may be facilitated by the proinflammatory status found in CF. We showed that CFTR malfunction helps intracellular infections and since the intracellular milieu itself may trigger the formation

of SCVs (63), it is possible that phenotypic switching further encourages the persistence of bacteria in the lungs of CF patients.

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REFERENCES

1. **Alexander, E. H., and M. C. Hudson.** 2001. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. Appl Microbiol Biotechnol **56:**361-366.
2. **Archer, G. L.** 1998. *Staphylococcus aureus*: a well-armed pathogen. Clin Infect Dis **26:**1179-1181.

3. **Atalla, H., C. Gyles, C. L. Jacob, H. Moisan, F. Malouin, and B. Mallard.** 2008. Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. Foodborne Pathog Dis **5:**785-799.
4. **Atalla, H., C. Gyles, and B. Mallard.** 2009. Persistence of a *Staphylococcus aureus* small colony variants (*S. aureus* SCV) within bovine mammary epithelial cells. Vet Microbiol **143:**319-328.
5. **Balwit, J. M., P. van Langevelde, J. M. Vann, and R. A. Proctor.** 1994. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J Infect Dis **170:**1033-1037.
6. **Brouillette, E., A. Martinez, B. J. Boyll, N. E. Allen, and F. Malouin.** 2004. Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*. FEMS Immunol Med Microbiol **41:**35-41.
7. **Canadian Cystic Fibrosis Foundation.** 2007. Patient data registry report. Toronton, ON, Canada.
8. **Cannon, C. L., M. P. Kowalski, K. S. Stopak, and G. B. Pier.** 2003. *Pseudomonas aeruginosa*-induced apoptosis is defective in respiratory epithelial cells expressing mutant cystic fibrosis transmembrane conductance regulator. Am J Respir Cell Mol Biol **29:**188-197.
9. **Cantin, A. M., G. Bilodeau, C. Ouellet, J. Liao, and J. W. Hanrahan.** 2006. Oxidant stress suppresses CFTR expression. Am J Physiol Cell Physiol **290:**C262-270.
10. **Cantin, A. M., J. W. Hanrahan, G. Bilodeau, L. Ellis, A. Dupuis, J. Liao, J. Zielenski, and P. Durie.** 2006. Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. Am J Respir Crit Care Med **173:**1139-1144.
11. **Carterson, A. J., K. Honer zu Bentrup, C. M. Ott, M. S. Clarke, D. L. Pierson, C. R. Vanderburg, K. L. Buchanan, C. A. Nickerson, and M. J. Schurr.** 2005. A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis. Infect Immun **73:**1129-1140.

12. **Cuny, C., A. Friedrich, S. Kozytska, F. Layer, U. Nübel, K. Ohlsen, B. Strommenger, B. Walther, L. Wieler, and W. Witte.** 2010. Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. Int J Med Microbiol **300:**109-117.
13. **Cystic Fibrosis Foundation.** 2008. Patient registry annual report. Washington, D.C.
14. **Di, A., M. E. Brown, L. V. Deriy, C. Li, F. L. Szeto, Y. Chen, P. Huang, J. Tong, A. P. Naren, V. Bindokas, H. C. Palfrey, and D. J. Nelson.** 2006. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. Nat Cell Biol **8:**933-944.
15. **Döring, G., and E. Gulbins.** 2009. Cystic fibrosis and innate immunity: how chloride channel mutations provoke lung disease. Cell Microbiol **11:**208-216.
16. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol **38:**1008-1015.
17. **Fang, X., Y. Song, J. Hirsch, L. J. Galietta, N. Pedemonte, R. L. Zemans, G. Dolganov, A. S. Verkman, and M. A. Matthay.** 2006. Contribution of CFTR to apical-basolateral fluid transport in cultured human alveolar epithelial type II cells. Am J Physiol Lung Cell Mol Physiol **290:**L242-249.
18. **Fleiszig, S. M., D. J. Evans, N. Do, V. Vallas, S. Shin, and K. E. Mostov.** 1997. Epithelial cell polarity affects susceptibility to *Pseudomonas aeruginosa* invasion and cytotoxicity. Infect Immun **65:**2861-2867.
19. **Goering, R. V., A. Bauernfeind, W. Lenz, and B. Przyklenk.** 1990. *Staphylococcus aureus* in patients with cystic fibrosis: an epidemiological analysis using a combination of traditional and molecular methods. Infection **18:**57-60.
20. **Goerke, C., and C. Wolz.** 2004. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. Int J Med Microbiol **294:**195-202.

21. **Grainger, C. I., L. L. Greenwell, D. J. Lockley, G. P. Martin, and B. Forbes.** 2006. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. Pharm Res **23:**1482-1490.

22. **Hunter, M. J., K. J. Treharne, A. K. Winter, D. M. Cassidy, S. Land, and A. Mehta.** 2010. Expression of wild-type CFTR suppresses NF-κB-driven inflammatory signalling. PLoS One **5:**e11598.

23. **Jarry, T. M., and A. L. Cheung.** 2006. *Staphylococcus aureus* escapes more efficiently from the phagosome of a cystic fibrosis bronchial epithelial cell line than from its normal counterpart. Infect Immun **74:**2568-2577.

24. **Joo, N. S., D. J. Lee, K. M. Winges, A. Rustagi, and J. J. Wine.** 2004. Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. J Biol Chem **279:**38854-38860.

25. **Joseph, T., D. Look, and T. Ferkol.** 2005. NF-κB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with *Pseudomonas aeruginosa*. Am J Physiol Lung Cell Mol Physiol **288:**L471-479.

26. **Kahl, B., M. Herrmann, A. S. Everding, H. G. Koch, K. Becker, E. Harms, R. A. Proctor, and G. Peters.** 1998. Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. J Infect Dis **177:**1023-1029.

27. **Kahl, B. C., G. Belling, P. Becker, I. Chatterjee, K. Wardecki, K. Hilgert, A. L. Cheung, G. Peters, and M. Herrmann.** 2005. Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infect Immun **73:**4119-4126.

28. **Krivan, H. C., D. D. Roberts, and V. Ginsburg.** 1988. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. Proc Natl Acad Sci U S A **85:**6157-6161.

29. **Langlois, C., and F. P. Gendron.** 2009. Promoting Mφ transepithelial migration by stimulating the epithelial cell $P2Y_2$ receptor. Eur J Immunol **39:**2895-2905.

30. **Lowy, F. D.** 2000. Is *Staphylococcus aureus* an intracellular pathogen? Trends Microbiol **8:**341-343.

31. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev **15:**194-222.

32. **Ma, T., J. R. Thiagarajah, H. Yang, N. D. Sonawane, C. Folli, L. J. Galietta, and A. S. Verkman.** 2002. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J Clin Invest **110:**1651-1658.

33. **Malachowa, N., A. Sabat, M. Gniadkowski, J. Krzyszton-Russjan, J. Empel, J. Miedzobrodzki, K. Kosowska-Shick, P. C. Appelbaum, and W. Hryniewicz.** 2005. Comparison of multiple-locus variable-number tandem-repeat analysis with pulsed-field gel electrophoresis, spa typing, and multilocus sequence typing for clonal characterization of *Staphylococcus aureus* isolates. J Clin Microbiol **43:**3095-3100.

34. **Mitchell, G., E. Brouillette, D. L. Séguin, A. E. Asselin, C. L. Jacob, and F. Malouin.** 2010. A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides. Microb Pathog **48:**18-27.

35. **Mitchell, G., M. Gattuso, G. Grondin, E. Marsault, K. Bouarab, and F. Malouin.** 2011. Tomatidine Inhibits the Replication of *Staphylococcus aureus* Small-Colony Variants in Cystic Fibrosis Airway Epithelial Cells. Antimicrob Agents Chemother **55:**1937-1945.

36. **Mitchell, G., C. A. Lamontagne, E. Brouillette, G. Grondin, B. G. Talbot, M. Grandbois, and F. Malouin.** 2008. *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. Mol Microbiol **70:**1540-1555.

37. **Mitchell, G., D. L. Séguin, A. E. Asselin, E. Déziel, A. M. Cantin, E. H. Frost, S. Michaud, and F. Malouin.** 2010. *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to Pseudomonas aeruginosa 4-hydroxy-2-heptylquinoline-N-oxide. BMC Microbiol **10:**33.

38. **Moisan, H., E. Brouillette, C. L. Jacob, P. Langlois-Bégin, S. Michaud, and F. Malouin.** 2006. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J Bacteriol **188:**64-76.

39. **Nickerson, C. A., T. J. Goodwin, J. Terlonge, C. M. Ott, K. L. Buchanan, W. C. Uicker, K. Emami, C. L. LeBlanc, R. Ramamurthy, M. S. Clarke, C. R. Vanderburg, T. Hammond, and D. L. Pierson.** 2001. Three-dimensional tissue assemblies: novel models for the study of *Salmonella enterica* serovar Typhimurium pathogenesis. Infect Immun **69:**7106-7120.

40. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol **48:**1429-1449.

41. **Oviedo-Boyso, J., J. G. Barriga-Rivera, J. J. Valdez-Alarcón, A. Bravo-Patiño, A. Cárabez-Trejo, M. Cajero-Juárez, and V. M. Baizabal-Aguirre.** 2008. Internalization of *Staphylococcus aureus* by bovine endothelial cells is associated with the activity state of NF-κB and modulated by the pro-inflammatory cytokines TNF-α and IL-1β. Scand J Immunol **67:**169-176.

42. **Palmer, M. L., S. Y. Lee, P. J. Maniak, D. Carlson, S. C. Fahrenkrug, and S. M. O'Grady.** 2006. Protease-activated receptor regulation of Cl- secretion in Calu-3 cells requires prostaglandin release and CFTR activation. Am J Physiol Cell Physiol **290:**C1189-1198.

43. **Perkins, N. D.** 2007. Integrating cell-signalling pathways with NF-κB and IKK function. Nat Rev Mol Cell Biol **8:**49-62.

44. **Plotkowski, M. C., S. de Bentzmann, S. H. Pereira, J. M. Zahm, O. Bajolet-Laudinat, P. Roger, and E. Puchelle.** 1999. *Pseudomonas aeruginosa* internalization by human epithelial respiratory cells depends on cell differentiation, polarity, and junctional complex integrity. Am J Respir Cell Mol Biol **20:**880-890.

45. **Proctor, R. A., C. von Eiff, B. C. Kahl, K. Becker, P. McNamara, M. Herrmann, and G. Peters.** 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol **4:**295-305.

46. **Ramesh Babu, P. B., A. Chidekel, L. Utidjian, and T. H. Shaffer.** 2004. Regulation of apical surface fluid and protein secretion in human airway epithelial cell line Calu-3. Biochem Biophys Res Commun **319:**1132-1137.

47. **Ratner, A. J., R. Bryan, A. Weber, S. Nguyen, D. Barnes, A. Pitt, S. Gelber, A. Cheung, and A. Prince.** 2001. Cystic fibrosis pathogens activate Ca^{2+}-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. J Biol Chem **276:**19267-19275.

48. **Riordan, J. R.** 2008. CFTR function and prospects for therapy. Annu Rev Biochem **77:**701-726.

49. **Rubin, B. K.** 2007. CFTR is a modulator of airway inflammation. Am J Physiol Lung Cell Mol Physiol **292:**L381-382.

50. **Sabat, A., J. Krzyszton-Russjan, W. Strzalka, R. Filipek, K. Kosowska, W. Hryniewicz, J. Travis, and J. Potempa.** 2003. New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. J Clin Microbiol **41:**1801-1804.

51. **Saiman, L., and A. Prince.** 1993. *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J Clin Invest **92:**1875-1880.

52. **Schröder, A., R. Kland, A. Peschel, C. von Eiff, and M. Aepfelbacher.** 2006. Live cell imaging of phagosome maturation in *Staphylococcus aureus* infected human endothelial cells: small colony variants are able to survive in lysosomes. Med Microbiol Immunol **195:**185-194.

53. **Sendi, P., and R. A. Proctor.** 2009. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends Microbiol **17:**54-58.

54. **Senn, M. M., M. Bischoff, C. von Eiff, and B. Berger-Bächi.** 2005. σ^B activity in a *Staphylococcus aureus hemB* mutant. J Bacteriol **187:**7397-7406.

55. **Sinha, B., P. P. Francois, O. Nüße, M. Foti, O. M. Hartford, P. Vaudaux, T. J. Foster, D. P. Lew, M. Herrmann, and K. H. Krause.** 1999. Fibronectin-binding

protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin $\alpha_5\beta_1$. Cell Microbiol **1**:101-117.

56. **Teichgräber, V., M. Ulrich, N. Endlich, J. Riethmüller, B. Wilker, C. C. De Oliveira-Munding, A. M. van Heeckeren, M. L. Barr, G. von Kürthy, K. W. Schmid, M. Weller, B. Tümmler, F. Lang, H. Grassme, G. Döring, and E. Gulbins.** 2008. Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nat Med **14**:382-391.
57. **Tenover, F. C., R. R. Vaughn, L. K. McDougal, G. E. Fosheim, and J. E. McGowan, Jr.** 2007. Multiple-locus variable-number tandem-repeat assay analysis of methicillin-resistant *Staphylococcus aureus* strains. J Clin Microbiol **45**:2215-2219.
58. **Tirouvanziam, R., S. de Bentzmann, C. Hubeau, J. Hinnrasky, J. Jacquot, B. Peault, and E. Puchelle.** 2000. Inflammation and infection in naive human cystic fibrosis airway grafts. Am J Respir Cell Mol Biol **23**:121-127.
59. **Tobe, M., Y. Isobe, H. Tomizawa, T. Nagasaki, H. Takahashi, T. Fukazawa, and H. Hayashi.** 2003. Discovery of quinazolines as a novel structural class of potent inhibitors of NF-κB activation. Bioorg Med Chem **11**:383-391.
60. **Tuchscherr, L., V. Heitmann, M. Hussain, D. Viemann, J. Roth, C. von Eiff, G. Peters, K. Becker, and B. Löffler.** *Staphylococcus aureus* Small-Colony Variants Are Adapted Phenotypes for Intracellular Persistence. J Infect Dis **202**:1031-1040.
61. **Tuchscherr, L., E. Medina, M. Hussain, W. Völker, V. Heitmann, S. Niemann, D. Holzinger, J. Roth, R. A. Proctor, K. Becker, G. Peters, and B. Löffler.** 2011. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med **3**:129-141.
62. **Vaudaux, P., P. Francois, C. Bisognano, W. L. Kelley, D. P. Lew, J. Schrenzel, R. A. Proctor, P. J. McNamara, G. Peters, and C. Von Eiff.** 2002. Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. Infect Immun **70**:5428-5437.

63. **Vesga, O., M. C. Groeschel, M. F. Otten, D. W. Brar, J. M. Vann, and R. A. Proctor.** 1996. *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. J Infect Dis **173:**739-742.

64. **Vij, N., S. Mazur, and P. L. Zeitlin.** 2009. CFTR is a negative regulator of NFκB mediated innate immune response. PLoS One **4:**e4664.

65. **von Eiff, C., C. Heilmann, R. A. Proctor, C. Woltz, G. Peters, and F. Götz.** 1997. A site-directed *Staphylococcus aureus hemB* mutant is a small-colony variant which persists intracellularly. J Bacteriol **179:**4706-4712.

66. **Wang, J. H., Y. J. Zhou, and P. He.** 2010. *Staphylococcus aureus* induces apoptosis of human monocytic U937 cells via NF-κB signaling pathways. Microb Pathog.

67. **Zander, J., S. Besier, S. H. Saum, F. Dehghani, S. Loitsch, V. Brade, and T. A. Wichelhaus.** 2008. Influence of dTMP on the phenotypic appearance and intracellular persistence of *Staphylococcus aureus*. Infect Immun **76:**1333-1339.

CHAPITRE 5

Interleukin-6 and interleukin-8 secretions by polarized airway epithelial cells infected by normal and small-colony variant *Staphylococcus aureus* strains are similar despite differences in infection levels.

5.1. Introduction de l'article

Le but de cette étude était d'évaluer la réponse inflammatoire enclenchée lors de l'infection de cellules épithéliales pulmonaires polarisées avec des souches normales et SCVs de *S. aureus*. Nous avons ici collaboré avec le Pr Brian G. Talbot et son étudiante Myriame Lafrance. J'ai effectué les essais d'infection de cellules et participé au dosage des cytokines dans les surnageants de culture cellulaire. J'ai rédigé l'ébauche de l'article, effectué la conception de la figure et participé à la correction du manuscrit jusqu'à sa publication.

Référence de l'article :

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5.2. Résumé en français de l'article

Les SCVs de *Staphylococcus aureus* peuvent efficacement infecter les phagocytes non professionnels et sont maintenant considérés comme des pathogènes intracellulaires facultatifs. L'habileté à se cacher à l'intérieur des cellules contribue vraisemblablement au développement d'infections chroniques à *S. aureus* tel que celles observées dans les voies respiratoires des patients FK. Des cellules pulmonaires humaines Calu-3 polarisées furent utilisées afin de confirmer que les SCVs persistent à l'intérieur des cellules épithéliales sans exacerber la réponse immunitaire innée. Tandis que toutes les souches de *S. aureus* étudiées induisaient significativement la sécrétion d'interleukine-6 (IL-6) et d'interleukine-8 (IL-8) par les Calu-3 48 heures suivant l'invasion cellulaire, cela n'était pas le cas de bactéries mortes. D'une façon surprenante, aucune différence dans la sécrétion de ces interleukines n'était détectée entre des cellules infectées avec des souches normales et SCVs malgré la différence marquée dans les niveaux d'infections. Cette étude supporte l'hypothèse que malgré leur habileté augmentée à persister à l'intérieur des cellules épithéliales, les SCVs n'activent pas la réponse immunitaire en comparaison aux souches normales. Les SCVs pourraient donc aider la perpétuation de l'infection sans toutefois exacerber la réponse immunitaire de l'hôte.

5.3. L'article scientifique

Interleukin-6 and interleukin-8 secretions by polarized airway epithelial cells infected by normal and small-colony variant *Staphylococcus aureus* strains are similar despite differences in infection levels

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Running title: IL-6 & IL-8 secretions by SCV infected cells.

Keywords: innate immunity, intracellular infections, cystic fibrosis, SCVs.

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ABSTRACT

Staphylococcus aureus small-colony variants (SCVs) can efficiently infect non-professional phagocytes and are often referred to as facultative intracellular pathogens. The ability to hide and persist within host cells is likely to contribute to the development of chronic *S. aureus* infections such as those observed in the lungs of cystic fibrosis patients. Polarized human pulmonary Calu-3 cells were used to confirm that *S. aureus* small-colony variants (SCVs) persist within epithelial cells without exacerbating the innate immune response. Whereas all studied *S. aureus* strains significantly induced the secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) by Calu-3 cells 48 hours after cellular invasion, dead bacteria did not. Surprisingly, no difference in the secretion of these interleukins was detected between cells infected with normal and SCV strains despite the marked difference in infection levels. This study supports the hypothesis that despite their increased ability to persist inside epithelial cells, SCVs do not over activate the host immune response in comparison to normal strains. SCVs may thus help to perpetuate infection without exacerbation of the host immune response.

Abbreviations: *agr*, accessory gene regulator; CF, cystic fibrosis; CFU, colony-forming unit; D.B., dead bacteria; FBS, fetal bovine serum; G-CSF, granulocyte colony-stimulating factor; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8;
INF-γ, interferon-γ; IP-10, INF-γ inducible protein 10kD; MCP-1, monocyte chemoattractant protein-1; PBS, phosphate buffered saline; SCVs (or SCV), small-colony variants; TNF-α, tumor necrosis factor-α.

MAIN TEXT

Strains forming pin-point colonies and called small-colony variants (SCVs) are often isolated from *Staphylococcus aureus* chronic infections such as those encountered in the lungs of cystic fibrosis (CF) patients and also from osteomyelitis, septic arthritis and infections of orthopedic devices. Several SCVs are respiratory deficient strains that show a dysfunctional electron transport system. Such characteristic affects the growth rate, disrupts the proton motive force and decreases the susceptibility to aminoglycoside antibiotics, and also alters the expression of several virulence factors [1]. SCVs are now known to have an increased ability to form biofilms [2, 3] and to infect non-professional phagocytes [4]. This may promote the development of chronic infections by shielding the bacteria against the host immune system and the action of some antibiotics [4-6].

The distinct virulence profile of SCVs may result from the inability of the bacteria to properly activate the accessory gene regulator (*agr*) quorum-sensing system and/or by a sustained activity of the alternative transcription factor sigma B [7-9]. Interestingly, *S. aureus* activation of the *agr* system has been associated with the production of an innate immune response in both endothelial [10] and airway epithelial [11] cells. It was also demonstrated that normal *S. aureus* strains cause an inflammatory response in endothelial cells whereas SCVs did not [12]. In light of these results, we conducted a study aimed to evaluate the inflammatory response triggered by the infection of polarized human airway epithelial cells with normal and SCV strains, since these cells constitute the first line of defense against lung pathogens [13].

We used Calu-3 cells grown at the air-interface as our infection model. These cells are known to have many features of polarized and differentiated airway epithelial cells when grown *in vitro* [14-16] and should thus help in the study of interactions between the airway epithelium

and *S. aureus*. The Calu-3 cell line (ATCC HTB 55) was cultured in Eagle's minimum essential medium supplemented with 0.1mM minimum essential medium nonessential amino acids, 1 mM of sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml of Fungizone and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 (Wisent, QC, Canada). Cell infection assays were performed as previously described with few modifications [8, 15, 16]. Briefly, cells were seeded at 1.5 x 10^5 cells/inserts on 12-wells Transwells (Fischer, ON, Canada) and cultured for 9 to 10 days in an air:liquid system. Approximately 1 × 10^6 Hoescht-stained nucleus/insert were then observed. The complete medium in the basal compartments was replaced by the invasion medium (1% FBS and no antibiotics) 18 h before assays. Inocula were prepared by suspending bacteria grown 20 h on brain heart infusion agar plates in ice-cold phosphate buffered saline (PBS). Bacteria were then washed three times in ice-cold PBS and suspended in the invasion medium supplemented with 0.5% bovine serum albumin at a density of approximately 4 x 10^8 colony-forming unit (CFU)/ml. Cells were washed twice with PBS and 250 μl of the bacterial suspension was added to the apical side of each insert. Invasion was allowed for 3 h, inserts were emptied and washed three times with PBS. Invasion medium supplemented with 20 μg/ml of lysostaphin (Sigma, ON, Canada) was then added to kill extracellular bacteria and the cells were further incubated for a varying amount of time. Following three washes with PBS, cells were detached with 100 μl of trypsin 0.25% and lysed for 10 min by the addition of 400 μl of water containing 0.05% of Triton X-100. Lysates were serially diluted 10-fold and plated on agar for bacterial CFU determination. The intracellular localization of bacteria was confirmed by fluorescence microscopy using the anti-*S. aureus* antibody AB20920 (Abcam, MA, USA) and the Olympus Fluoview FV 300 confocal system [15, 16].

The strains CF07-L and CF07-S were previously compared for their ability to infect polarized Calu-3 cells [16]. CF07-L and CF07-S are genetically related *S. aureus* strains co-isolated from a CF patient, which respectively have a normal and a SCV phenotype [2]. Infection kinetics revealed no significant differences in the level of intracellular CFU recovered from

Calu-3 cells infected with these strains 3, 9 and 24 h post-invasion, whereas a striking difference was found 48 h post-invasion [16]. This result was confirmed with another pair of genetically-related non-SCV/SCV strains (CF1A-L and CF1D-S, respectively) co-isolated from another CF patient [2]. Fig.1A confirms that CFU recovered from cells infected by the SCV of both pairs of strains are markedly higher than those of their normal counterpart 48 h post-invasion (more than a 2 $\log_{10}$ increase in magnitude).

In order to evaluate the immune response of Calu-3 cells to *S. aureus* infections, combinations of flowcytomix™ simplex kits were used according to the recommendations of the manufacturer (eBioscience, San Diego, CA). The extent of granulocyte colony-stimulating factor (G-CSF), interleukin-1β (IL-1β), IL-6, IL-8, interferon- γ (INF-γ), INF-γ inducible protein 10kD (IP-10), monocyte chemoattractant protein-1(MCP-1) and tumor necrosis factor-α (TNF-α) secretion by Calu-3 cells infected by *S. aureus* at 3, 9, 24 and 48 hours post-invasion was initially evaluated to establish detectable and reproducible thresholds (data not shown). Only IL-6 and IL-8 secretions at 48 h post-invasion were selected to compare the effect of different strains on the activation of the immune response because only these cytokines were significantly induced during the infection of Calu-3 cells by *S. aureus*, although the biological significance of the low IL-6 secretion levels in these infected cells may be marginal. According to the manufacturer, the sensitivity of the method was of 1.2 and 0.5 pg/ml for IL-6 and IL-8, respectively. As expected, treatment of cells with 100 ng/ml of TNF-α or 10 μg/ml of LPS for 48 h also stimulated IL-6 and IL-8 secretion (data not shown). The induction of the pro-inflammatory mediators IL-6 and IL-8 in epithelial cells infected by *S. aureus* has also been reported by others [11, 13, 17-19] and is thought to constitute a critical part of the lung immune response to bacterial pathogens [13].

The secretion of IL-6 and IL-8 was then measured from Calu-3 cells infected with the normal and SCV strains CF07-L, CF07-S, CF1A-L and CF1D-S, and compared to that from non-

infected Calu-3 cells. In addition, the immune response of cells exposed to approximately $1x10^8$ CFU of heat inactivated CF07-L bacteria (30 min at 72°C) was evaluated since live or dead *S. aureus* may not trigger the same response in host cells [20, 21]. Fig. 1B and 1C show that all studied *S. aureus* strains induced IL-6 and IL-8 secretion in airway epithelial cells 48 h post-invasion in comparison to uninfected cells (one-way ANOVA followed by the Dunnett's post test), whereas dead bacteria had no effect on the cells (unpaired *t*-test). Surprisingly, no difference was observed in the extent of IL-6 and IL-8 secretion by airway epithelial cells despite their being infected with different *S. aureus* strains (one-way ANOVA followed by the Tukey's post test) and notwithstanding the markedly greater ability of SCV strains CF07-S and CF1D-S to persist 48 h post-invasion in comparison to their normal counterparts. Noteworthy, normal colony-forming bacteria were recovered at a low frequency 48 hours post-invasion from cells infected with SCVs. It is thus possible that the induction of IL-6 and IL-8 secretions in SCV infected cells may have arisen by phenotypic switching during intracellular infections especially that the SCV strains used in this study are clinical isolates that have kept the natural ability to revert back to the normal phenotype.

This study supports the hypothesis that SCVs do not particularly activate the host immune system despite their marked intracellular persistence [4, 12]. This may help to explain why some infections caused by SCVs can be sustained asymptomatically for many years [22-24]. Tuchscherr *et al.* [25] have recently proposed that the switch from the normal to the SCV phenotype and *vice versa* could be an integral part of the infection process. It is likely that the SCV phenotype can confer the ability to *S. aureus* to remain hidden inside non-professional phagocytes for some periods of time until reversion to normal phenotype occurs and a new acute phase of infection begins. Therapeutic tools to tackle both the normal and persistent phenotypes seem implicitly needed [15].

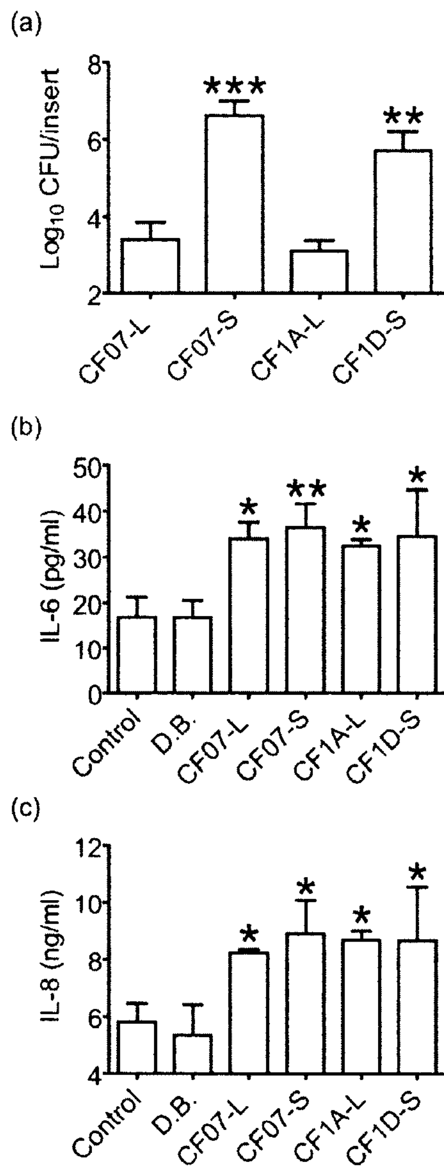

FIGURE 1. IL-6 and IL-8 secretions by polarized airway epithelial cells infected with normal and SCV strains are similar despite marked differences in infection levels. (A) Bacterial CFU recovered 48 h post-invasion from polarized Calu-3 cells infected with the *S. aureus* SCV strains CF07-S and CF1D-S and their normal counterparts CF07-L and CF1A-L, respectively. Significant differences between CFU recovered from cells for normal and SCV strains of each

pairs are indicated (**, $P < 0.01$; ***, $P < 0.001$; unpaired *t*-test). Figures 1B and 1C show the concentrations of IL-6 and IL-8 in the basal compartment of Transwells seeded with polarized Calu-3 cells, respectively. Cells were either non-infected (untreated control), treated with dead bacteria (D.B.) or infected with the various strains CF07-L, CF07-S, CF1A-L and CF1D-S. The medium from the basal compartment of the Transwell was collected 48 h post-invasion for the measurement of IL-6 and IL-8 concentrations. Significant differences between the control and the infected conditions are shown (*, $P < 0.05$; **, $P < 0.01$; one-way ANOVA followed by the Dunnett's post test). No difference between strains was revealed by a one-way ANOVA followed by the Tukey's post test. Results are from three independent experiments performed in duplicate.

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REFERENCES

1. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, *et al.* (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4: 295-305.
2. Mitchell G, Séguin DL, Asselin AE, Déziel E, Cantin AM, *et al.* (2010) *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. BMC Microbiol 10: 33.

3. Singh R, Ray P, Das A , Sharma M (2010) Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant. J Med Microbiol 59: 521-7.
4. Sendi P, Proctor RA (2009) *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends Microbiol 17: 54-8.
5. Stewart PS (2002) Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol 292: 107-13.
6. Galli J, Ardito F, Calo L, Mancinelli L, Imperiali M, *et al.* (2007) Recurrent upper airway infections and bacterial biofilms. J Laryngol Otol 121: 341-4.
7. Kahl BC, Belling G, Becker P, Chatterjee I, Wardecki K, *et al.* (2005) Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infect Immun 73: 4119-26.
8. Moisan H, Brouillette E, Jacob CL, Langlois-Bégin P, Michaud S, *et al.* (2006) Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J Bacteriol 188: 64-76.
9. Mitchell G, Lamontagne CA, Brouillette E, Grondin G, Talbot BG, *et al.* (2008) *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. Mol Microbiol 70: 1540-55.
10. Grundmeier M, Tuchscherr L, Bruck M, Viemann D, Roth J, *et al.* (2010) Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells. J Infect Dis 201: 871-80.
11. Ratner AJ, Bryan R, Weber A, Nguyen S, Barnes D, *et al.* (2001) Cystic fibrosis pathogens activate Ca^{2+}-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. J Biol Chem 276: 19267-75.
12. Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, *et al.* (2010) *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis 202: 1031-40.

13. Gómez MI, Prince A (2008) Airway epithelial cell signaling in response to bacterial pathogens. Pediatr Pulmonol 43: 11-9.

14. Grainger CI, Greenwell LL, Lockley DJ, Martin GP, Forbes B (2006) Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. Pharm Res 23: 1482-90.

15. Mitchell G, Gattuso M, Grondin G, Marsault E, Bouarab K, *et al.* (2011) Tomatidine Inhibits Replication of *Staphylococcus aureus* Small-Colony Variants in Cystic Fibrosis Airway Epithelial Cells. Antimicrob Agents Chemother 55: 1937-45.

16. Mitchell G, Grondin G, Bilodeau G, Cantin AM , Malouin F (2011) Infection of Polarized Airway Epithelial Cells by Normal and Small-Colony Variant Strains of *Staphylococcus aureus* is Increased in Cells with Abnormal CFTR function and is influenced by NF-κB. Infect Immun. Epub ahead of print.

17. Sachse F, Becker K, von Eiff C, Metze D , Rudack C (2010) *Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells *in vitro*. Allergy.

18. Fragaki K, Kileztky C, Trentesaux C, Zahm JM, Bajolet O, *et al.* (2006) Downregulation by a long-acting β2-adrenergic receptor agonist and corticosteroid of *Staphylococcus aureus*-induced airway epithelial inflammatory mediator production. Am J Physiol Lung Cell Mol Physiol 291: L11-8.

19. Escotte S, Al Alam D, Le Naour R, Puchelle E, Guenounou M, *et al.* (2006) T cell chemotaxis and chemokine release after *Staphylococcus aureus* interaction with polarized airway epithelium. Am J Respir Cell Mol Biol 34: 348-54.

20. Haslinger-Löffler B, Kahl BC, Grundmeier M, Strangfeld K, Wagner B, *et al.* (2005) Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells. Cell Microbiol 7: 1087-97.

21. Strindhall J, Lindgren PE, Lofgren S , Kihlstrom E (2002) Variations among clinical isolates of *Staphylococcus aureus* to induce expression of E-selectin and ICAM-1 in human endothelial cells. FEMS Immunol Med Microbiol 32: 227-35.

22. Proctor RA, van Langevelde P, Kristjansson M, Maslow JN , Arbeit RD (1995) Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin Infect Dis 20: 95-102.

23. Kipp F, Ziebuhr W, Becker K, Krimmer V, Höβ N, *et al.* (2003) Detection of *Staphylococcus aureus* by 16S rRNA directed *in situ* hybridisation in a patient with a brain abscess caused by small colony variants. J Neurol Neurosurg Psychiatry 74: 1000-2.

24. von Eiff C, Becker K, Metze D, Lubritz G, Hockmann J, *et al.* (2001) Intracellular persistence of *Staphylococcus aureus* small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with darier's disease. Clin Infect Dis 32: 1643-7.

25. Tuchscherr L, Medina E, Hussain M, Volker W, Heitmann V, et al. (2011) *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med 3: 129-41.

CHAPITRE 6

Tomatidine inhibits the replication of *Staphylococcus aureus* small-colony variants in cystic fibrosis airway epithelial cells.

6.1. Introduction de l'article

Le but de cette étude était d'évaluer l'activité antibactérienne de la tomatidine contre les SCVs de *S. aureus* et d'investiguer son mode d'action. En collaboration avec le Pr Kamal Bouarab, l'étudiante Mariza Gattuso du laboratoire du Pr François Malouin a débuté un projet adressant l'effet de la tomatidine sur l'expression de gènes de virulence chez les souches normales de *S. aureus*. Lorsque j'ai été recruté afin de contribuer aux travaux de cette étudiante, j'ai découvert l'activité bactériostatique de la tomatidine contre les SCVs. J'ai effectué toutes les expériences de microbiologie, les essais de susceptibilité aux antibiotiques et les essais d'infections de cellules. Gilles Grondin a effectué les expériences de microscopie et le Pr Éric Marsault est un collaborateur au projet. J'ai rédigé l'ébauche de l'article, effectué la conception des figures et participé à la correction du manuscrit jusqu'à sa publication. À noter que ce projet a mené au dépôt d'un brevet (voir Annexe 2, Résumé 3).

Référence de l'article :

Mitchell, G., Gattuso, M., Grondin, G., Marsault, É., Bouarab, K., and Malouin, F. (2011). Tomatidine inhibits replication of *Staphylococcus aureus* small-colony variants in cystic fibrosis airway epithelial cells. Antimicrob. Agents Chemother. *55*:1937-1945.

6.2. Résumé en français de l'article

Les SCVs sont souvent associés aux infections chroniques à *Staphylococcus aureus* tel que celles rencontrées chez les patients FK. Nous rapportons ici que la tomatidine, la forme aglycone du métabolite secondaire de plante tomatine, a une activité inhibitrice de la croissance efficace contre les SCVs (CMI de 0.12 μg/ml) tandis que la croissance des souches normales de *S. aureus* n'était pas significativement altérée par la tomatidine (MIC >16 μg/ml). L'action spécifique de la tomatidine était bactériostatique contre les SCVs et était clairement associée à leur chaîne de transport des électrons dysfonctionnelle puisque la présence de l'inhibiteur de transport d'électrons 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) rendait les souches normales de *S. aureus* susceptibles à la tomatidine. À l'inverse, la complémentation de la déficience respiratoire des SCVs conférait une résistance à la tomatidine. À une concentration correspondante à sa CMI contre les SCVs, la tomatidine causait une réduction générale de la biosynthèse de macromolécules et affectait particulièrement l'incorporation de leucine radiomarquée dans les protéines de *S. aureus* lorsqu'en présence du HQNO. De plus, la tomatidine inhibait la réplication intracellulaire d'un SCV clinique dans des cellules épithéliales au phénotype FK. Nos résultats suggèrent que la tomatidine pourrait éventuellement être utile en thérapie combinée avec d'autres antibiotiques traditionnels afin d'éliminer les formes persistantes de *S. aureus*.

6.3. L'article scientifique

Tomatidine inhibits the replication of *Staphylococcus aureus* small-colony variants in cystic fibrosis airway epithelial cells

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Running title: Tomatidine inhibits the replication of SCVs in CF cells.

Keywords: Chronic infections, small-colony variants, antimicrobials, pathogenesis, saponins.

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ABSTRACT

Small-colony variants (SCVs) are often associated with chronic *Staphylococcus aureus* infections such as those encountered by cystic fibrosis (CF) patients. We report here that tomatidine, the aglycon form of the plant secondary metabolite tomatine, has a potent growth inhibitory activity against SCVs (MIC of 0.12 μg/ml) whereas the growth of normal *S. aureus* strains was not significantly altered by tomatidine (MIC >16 μg/ml). The specific action of tomatidine was bacteriostatic for SCVs and was clearly associated to their dysfunctional electron transport system as the presence of the electron transport inhibitor 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) caused normal *S. aureus* strains to become susceptible to tomatidine. Inversely, complementation of SCVs' respiratory deficiency conferred resistance to tomatidine. Tomatidine showed a general reduction of macromolecular biosynthesis but more specifically affected incorporation of radiolabeled leucine in proteins of HQNO-treated *S. aureus* at a concentration corresponding to the MIC against SCVs. Furthermore, tomatidine inhibited the intracellular replication of a clinical SCV in polarized CF-like epithelial cells. Our results suggest that tomatidine may eventually find some use in combination therapy with other traditional antibiotics in order to eliminate persistent forms of *S. aureus*.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that can affect several hosts, organs and body sites and may become both life-threatening as well as chronic (2, 14). The ability of *S. aureus* to cause chronic infections is thought to be helped by the formation of biofilms and the persistence of the bacterium within non-phagocytic host cells, which may both offer protection against the host immune system and the action of antibiotics (1, 7, 12, 44).

Small-colony variants (SCVs) are known to form biofilms (27, 29, 42, 43) and to persist within non-phagocytic host cells (39). SCVs have a dysfunctional oxidative metabolism causing an alteration in the expression of virulence factors, a slow growth and a loss of colony pigmentation (33). This dysfunctional oxidative metabolism was associated to a decreased susceptibility to aminoglycosides because these antibiotics require the proton-motive force in order to penetrate the bacterium (8). This respiratory deficiency is often caused by mutations affecting the electron-transport system and several SCV isolates are auxotrophs for either hemin or menadione, which are needed to synthesize electron-transport system components. SCVs are often isolated from chronic infections, as in the case of lung infections in cystic fibrosis (CF) patients but also from osteomyelitis, septic arthritis, bovine mastitis and infection of orthopaedic devices (3, 30, 33). It was recently shown that switching from the normal to the SCV phenotype and than back to the normal phenotype *in vivo* is an integral part of the pathogenesis of *S. aureus*, a phenomenon that may be involved in the establishment of chronic infections (46).

Nosocomial and community-acquired infections caused by bacteria that are resistant to antibiotics represent an increasingly important public health concern. One of the reasons explaining the spread of antibiotic resistance resides in that the current arsenal of antibiotics

was largely designed on limited chemical scaffolds with only few innovations since the 1980s, leaving an opportunity for pathogens to develop and spread mechanisms of resistance worldwide (40, 45). There are now numerous reports of plant products providing antibiotic activities against a wide variety of pathogenic bacteria (10, 15, 34).

Several species of plants accumulate sterol and triterpene antimicrobials termed saponins (31). These molecules are constitutively produced in the plant and play an important role in the host defence against pathogenic insects and microbes. Among these saponins, tomatine is a steroidal glycoalkaloid that presents some antimicrobial action against yeast and a variety of microbes (4, 11, 31, 37). Many tomato fungal pathogens produce extracellular enzymes, commonly referred to as tomatinases, that are able to detoxify the α-tomatine (23, 35, 36). As an example, *Fusarium oxysporum* f. sp. *lycopersici* was reported to cleave α-tomatine into its aglycon (tomatidine) and tetrasaccharide moieties (lycotetraose), which by-products have little to no antifungal activity against the pathogen (36, 41). Some of our recent work on bacteria exposed to plant products shed light on the bioactivity of tomatidine against *S. aureus* and suggested a possible application for this molecule as a virulence attenuator for typical *S. aureus* strains (6) and, more recently, for SCVs (28). The chemical structures of tomatine and tomatidine are shown in Fig.1.

The aim of this study was to evaluate the antibacterial activity of tomatidine against *S. aureus* SCVs and to investigate its mode of action.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Staphylococcus aureus ATCC 29213, Newbould (ATCC 29740), and cystic fibrosis isolates CF07-L and CF1A-L were used as representatives of the normal phenotype whereas the strains NewbouldΔ*hemB* and cystic fibrosis isolates CF07-S and CF1D-S were used as SCV representatives. NewbouldΔ*hemB* was generated from Newbould by disrupting the *hemB* gene with the *ermA* cassette by homologous recombination (7). CF07-L/CF07-S and CF1A-L/CF1D-S are genetically related pairs of strains co-isolated from cystic fibrosis patients (29, 30). The dysfunctional oxidative metabolism of NewbouldΔ*hemB* and CF07-S can be complemented by hemin and menadione, respectively (29). Except where otherwise stated, bacteria were grown in brain heart infusion (BHI) broth (BD, Mississauga, ON, Canada).

Chemical reagents and antibiotics

Menadione (Sigma, Oakville, ON, Canada) was solubilized in DMSO at a concentration of 10 mg/ml and used at 1 µg/ml whereas hemin (Sigma) was solubilized in 1.4 M NH_4OH at a concentration of 10 mg/ml and used at 5 µg/ml. The electron transport inhibitor 4-hydroxy-2-heptylquinoline-N-oxide, HQNO (AXXORA, San Diego, CA, USA), was solubilized in DMSO at a concentration of 5 mg/ml and used at 20 µg/ml. Tomatidine, gentamicin, vancomycin, erythromycin, ciprofloxacin, oxacillin, rifampicin and norfloxacin were from Sigma whereas tomatine and chloramphenicol were from ICN biomedicals (Irvine, CA, USA) and Fisher (Ottawa, ON, Canada), respectively. Tomatine and tomatidine were solubilized at 10 mg/ml and 2 mg/ml in DMSO, respectively. Gentamicin, vancomycin, ciprofloxacin, oxacillin and norfloxacin were solubilized in water at 10 mg/ml. NaOH 2.5 and 0.1 N solutions were used during the solubilization of ciprofloxacin and norfloxacin, respectively. Erythromycin and chloramphenicol were solubilized at 10 mg/ml in 1:1 water:ethanol. Rifampicin was solubilized at 10 mg/ml in methanol.

Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by a broth microdilution technique, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (9), except that the incubation period was extended to 48 h and that the medium used was BHI in order to allow SCVs to reach maximal growth as previously described (3, 29). We confirmed that the MICs obtained against ATCC 29213 for all antibiotics tested were similar in BHI and in cation-adjusted Mueller-Hinton broth (CAMHB) (BD) to insure that the use of BHI did not influence results. The MICs of the control antibiotics used in the macromolecular synthesis assays were determined in CAMHB.

Time-kill experiments

Time-kill experiments were performed in order to determine whether the effect of compounds was bacteriostatic or bactericidal. Bacteria were inoculated at $\sim 5x10^5$ CFU/ml in BHI in the absence or presence of antibiotics at the specified concentrations (see figure legends). At several time points at 35°C (225 RPM), bacteria were sampled, serially diluted and plated on tryptic soy agar (TSA) for CFU determinations. Plates were incubated for 24 or 48 h at 35°C for normal and SCV strains, respectively.

Macromolecular biosynthesis assays

The complete defined medium (CDM) was used for macromolecular biosynthesis assays. CDM was constituted of the following chemicals per liter: 5 g glucose, 50 mg $MgSO_4$, 7 g K_2HPO_4, 2 g KH_2PO_4, 0.5 g of Na-Citrate dihydrate, 1 g $(NH_4)_2SO_4$, 1 mg thiamine, 1.2 mg niacin, 0.25 mg calcium pantothenate, 0.005 mg of biotin, 10 mg of L-tryptophan, 5 mg adenine, 5 mg guanine, 5 mg cytosine, 5 mg uracil, 100 mg L-glutamic acid, 90 mg L-aspartic acid, 80 mg L-proline, 50 mg L-arginine, 50 g glycine, 50 mg L-lysine, 60 mg L-alanine, 30 mg L-serine, 20 mg L-cysteine, 10 mg L-methionine, 50 mg L-tyrosine, 40 mg L-phenylalanine, 20 mg L-histidine, 30 mg L-threonine, 30 mg L-isoleucine, 80 mg L-valine, 90

mg L-leucine and 20 mg thymine. The medium CDM-LEU had 22.5 mg/l of L-Leucine instead of 90 mg/l whereas the medium CDM-ALA had 15 mg/l of L-alanine instead of 60 mg/l.

Protein, DNA, RNA and cell wall peptidoglycan biosynthesis were evaluated by measuring the incorporation of the appropriate radiolabeled precursors into bacteria prior to treatment with trichloroacetic acid (TCA). Inocula were prepared by incubating bacteria overnight at 35°C (225 RPM) in the CDM medium. Cultures were then adjusted to an optical density at 600nm (A_{600nm}) of 0.1 and grown until an A_{600nm} of 0.3 in CDM, CDM-LEU (protein) or CDM-ALA (cell wall) was achieved. An amount of 3µCi/ml of [^{3}H]leucine, 1µCi/ml of [^{3}H]thymine, 1µCi/ml of [^{3}H]uridine or 2µCi/ml [^{3}H]D-alanine was added to aliquots of cultures in presence of the different antimicrobial compounds in order to evaluate protein, DNA, RNA or cell wall peptidoglycan synthesis, respectively. The incorporation of [^{3}H]-molecules into macromolecules were allowed for 45 min for the protein and cell wall assays and for 35 min for the DNA and RNA assays. Cold 10% TCA was then added to all samples to stop the incorporation and precipitate macromolecules for 1 h on ice. All samples were filtered through a glass microfiber filter (Piscataway, NJ, USA) by using a dot-blot filtration system. Each filter was washed with 100 µl of 10% TCA containing 1.5 M NaCl and 100 µl of 10% TCA. Filters were dried overnight and their radioactivity was measured in a liquid scintillation counter. MICs of the control antibiotics chloramphenicol (protein), norfloxacin (DNA), rifampicin (RNA) and vancomycin (cell wall) against *S. aureus* ATCC 29213 were 8-16, 1, 0.008-0.015 and 0.5-1 µg/ml, respectively.

Cell culture

The CF-like human airway epithelial cells shCFTR (32), derived from the Calu-3 cell line (ATCC HTB 55), were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 0.1mM MEM nonessential amino acids, 1 mM of sodium pyruvate, 100

U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml of Fungizone and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. For routine culture, 4 μg/ml of puromycin was added to culture media. All cell culture reagents were purchased from Wisent (St-Bruno, QC, Canada).

Cell infection assays

Cell infection assays were performed as previously described with few adaptations for the Transwells system (26, 30). Cells were seeded at 2.5 x 10^5 cells/inserts on 12-well Transwell plates and cultured for 9 to 10 days in an air:liquid system. The complete medium in the basal compartment was replaced by the invasion medium (1% FBS and no antibiotics) 18 h before assays. Inocula were prepared by suspending bacteria grown 20 h on BHIA plates in ice-cold PBS. Bacteria were then washed three times in ice-cold PBS and suspended in the invasion medium supplemented with 0.5% BSA at a density of approximately 4 x 10^8 CFU/ml. Cells were washed twice with PBS and 250 μl of bacterial suspension were apically added to each insert. Invasion was allowed for 3 h, inserts were emptied and washed three times with PBS. Invasion medium supplemented with 20 μg/ml of lysostaphin (Sigma) was then added to kill extracellular bacteria and the cells were further incubated 24 or 48 h in presence of lysostaphin. DMSO or the different concentrations of tomatidine were added after invasion. At 24 h post-internalization for the 48-h assays, cells were washed once with PBS and the invasion medium supplemented with lysostaphin, DMSO and/or tomatidine was replaced. Fresh invasion medium supplemented with lysostaphin was also added 1 h before cell lysis to ensure that only intracellular bacteria were counted. Following three washes with PBS, cells were detached with 100 μl of trypsin 0.25% and lyzed for 10 min by the addition of 400 μl of water containing 0.05% of Triton X-100. Lysates were serially diluted 10-fold and plated on agar for CFU determination. Plates were incubated for 24 or 48 h at 35°C for normal and SCV strains, respectively.

Fluorescence microscopy

Cells on inserts were fixed 120 min in 4% paraformaldehyde in PBS. They were then permeabilized using a 5-min treatment with 50% methanol at -20°C followed by a 10-min incubation at room temperature in PBS supplemented with 50 mM glycine, 0.06% saponin from quillaja bark (Sigma), 0.06% Tween-20, 0.5% NP40 and 0.5% Triton X-100 (PBSP). The permeabilized cells were then incubated 30 min with image-iT® FX signal enhancer (Invitrogen, Burlington, ON, Canada). *S. aureus* was probed with the rabbit antibody AB20920 (Abcam, MA, USA) 1:250 and F actin was stained with AlexaFluor 488 Phalloidin (Invitrogen) 1:20 in PBS with 2% normal goat serum, 2% BSA and 0.45 % of fish gelatin (PBSB) for 180 min at room temperature. After five washes in PBS with 0.01% saponin and 0.01% Tween-20 (PBSD), primary antibodies were detected by an incubation of 90 min at room temperature with AlexaFluor 555 goat anti-rabbit IgG (Invitrogen) at a dilution of 1:1000 in PBSB. The DNA was stained 10 min with Hoechst (Invitrogen) at 1:5000 after five washes in PBSD and five washes in water. Inserts were washed five additional times in water and were mounted in Prolong Gold anti-fade reagent (Invitrogen). Pictures were taken using an Olympus Fluoview FV 300 confocal system or a Zeiss microscope with the Apotome system.

Effect of the combination of tomatidine and gentamicin

Bacteria were inoculated at ~$5x10^5$ CFU/ml in BHI in absence or presence of gentamicin and/or tomatidine at 4 and 0.12 µg/ml, respectively. Cultures were incubated 48 h at 35°C/225 RPM and pictures were taken. A_{600nm} of the different cultures were also measured.

Statistical analysis

Statistical analyses were carried out with the GraphPad Prism Software (v.5.00). Statistical tests used for the analysis of each experiment are specified in figure legends.

RESULTS

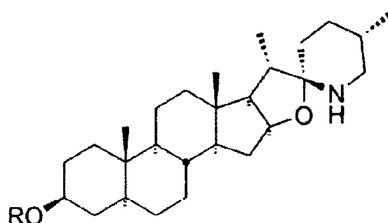

FIGURE 1. Structures of tomatidine (TO) and tomatine (TN). For TO, R= H. For TN, R= lycotetraose.

Tomatidine is a potent inhibitor of SCVs. Our previous works showed that although tomatidine (TO, Fig. 1) alters the expression of some virulence factors in normal *S. aureus* strains, it allows the growth of those bacteria at concentrations up to 128 µg/ml (6). After having observed that tomatidine also had the ability to inhibit the production of biofilms by *S. aureus* SCVs (28), we were interested to determine whether this result was attributable to a direct antimicrobial effect of tomatidine on SCVs. Table 1 shows the MICs of tomatidine, tomatine and control antibiotics (gentamicin, vancomycin, erythromycin, ciprofloxacin and oxacillin) against normal (ATCC 29213, Newbould, CF07-L and CF1A-L) and SCV (NewbouldΔ*hemB*, CF07-S and CF1D-S) strains. Remarkably, the MIC of tomatidine against all SCVs was 0.12 µg/ml whereas no MIC was measurable for normal strains. Also, no MIC was observed for tomatine, the lycotetraose-substituted derivative of tomatidine, against SCVs. MICs of gentamicin for the different strains were in accordance with the known decreased susceptibility of SCVs to aminoglycosides (33). The MIC of erythromycin against NewbouldΔ*hemB* (>16 µg/ml) is explained by the insertion of *ermA* in the genome of this strain (see Materials and Methods). MICs obtained for the other control antibiotics were in the expected CLSI ranges and did not seem to vary significantly among strains, except maybe for

ciprofloxacin MICs which were slightly lower for SCV strains. These results demonstrated that tomatidine specifically inhibits the growth of SCV strains.

TABLE 1. Susceptibility (MIC in µg/ml) of normal and SCV *S. aureus* strains to tomatidine, tomatine and control antibiotics with or without the presence of HQNO.

Strain[a]	HQNO[b]	TO	TN	GEN	VAN	ERY	CIP	OXA
ATCC29213	-	>16	>16	1	2	0.12-0.25	0.5	0.12-0.25
	+	0.12-0.25	>16	4	2	0.25	0.25	0.12
Newbould	-	>16	>16	0.5-1	1	0.25	0.25-0.5	0.06-0.12
NewbouldΔ*hemB*	-	0.12	>16	4-8	2	>16	0.12-0.25	0.03-0.06
CF07-L	-	>16	>16	1-2	2	0.25	0.5	0.06-0.12
	+	0.5	>16	4	2	0.25	0.25	0.06-0.12
CF07-S	-	0.12	>16	8	2	0.12	0.12	0.06-0.12
	+	0.12	>16	4-8	2	0.06-0.12	0.12	0.06-0.12
CF1A-L	-	>16	>16	1-2	1-2	0.25	0.5	0.25
CF1D-S	-	0.12	>16	8	2	0.12	0.12	0.06-0.12

[a]ATCC29213, Newbould, CF07-L and CF1A-L are normal strains whereas NewbouldΔ*hemB*,CF07-S and CF1D-S are SCVs.
[b]4-hydroxy-2-heptylquinoline-N-oxide (HQNO) was used at 20 µg/ml.
TO: tomatidine, TN: tomatine, GEN: gentamicin, VAN: vancomycin, ERY: erythromycin, CIP: ciprofloxacin, OXA: oxacillin.

Inhibition of the electron transport system by HQNO sensitizes normal strains to tomatidine. We used 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) to inhibit the electron transport system of normal strains and to generate the SCV phenotype (17, 20, 29). Table 1 shows MICs of tomatidine, tomatine and control antibiotics (gentamicin, vancomycin, erythromycin, ciprofloxacin and oxacillin) against the normal strains ATCC 29213 and CF07-L as well as the SCV CF07-S in the presence of 20 µg HQNO/ml. HQNO allowed tomatidine to generate a growth inhibitory effect against normal strains similar to that was observed against SCVs. HQNO did not however alter the susceptibility of SCVs to any other antibiotics. HQNO also decreased the susceptibility of normal strains against the aminoglycoside gentamicin, further supporting that the effect of HQNO on normal strains generates the SCV phenotype. Accordingly, subinhibitory concentrations of the proton motive force uncoupler carbonyl cyanide *m*-chlorophenylhydrazone also caused ATCC 29213 to become susceptible

to the growth inhibitory activity of tomatidine (data not shown) and decreased the susceptibility to gentamicin as previously reported (25). These results confirm that tomatidine possesses a specific antibacterial activity against SCVs because of their defective electron transport system.

Tomatidine is bacteriostatic against SCVs. We performed experiments in order to determine whether tomatidine is bactericidal or bacteriostatic against SCVs. Fig. 2A shows that tomatidine at concentrations up to 4 μg/ml is not able to completely kill SCVs (Newbould*ΔhemB*, CF07-S and CF1D-S) in culture, but clearly inhibited their growth in comparison to the normal strains Newbould, CF07-L and CF1A-L. Noteworthy, the susceptibility of the hemin-dependent SCV Newbould*ΔhemB* and menadione-dependent SCV CF07-S to tomatidine was abolished in the presence of hemin and menadione, respectively (also Fig. 2A), which again confirmed that a defective electron transport is required for the antibacterial activity of tomatidine. The antibacterial activities of tomatidine and control antibiotics (erythromycin and ciprofloxacin) against normal and SCV strains as a function of time are presented in Fig. 2B and 2C, respectively. The antibacterial activity of tomatine against the SCV strain was also evaluated (TN in Fig. 2C). Fig. 2C clearly demonstrates that the presence of tomatidine at 0.250 μg/ml (2×MIC) induced bacteriostasis in SCVs whereas it does not affect the growth of normal strains (Fig. 2B). Overall, our results demonstrate that tomatidine (and not tomatine) has a specific bacteriostatic activity against SCVs.

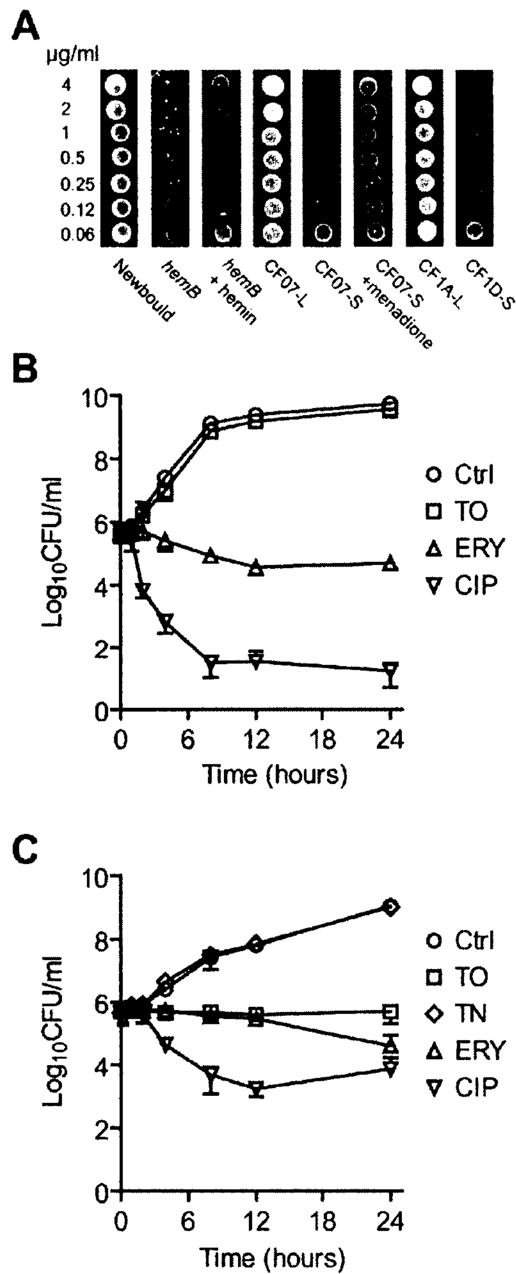

FIGURE 2. Tomatidine is bacteriostatic against SCVs, but not against normal strains. (A) Samples (10 µl) from cultures treated with various concentrations of tomatidine following the antibiotic susceptibility testing procedure (see Materials and Methods) were spotted on agar

plates and photographed. (B) Time-kill experiments showing the effect of tomatidine (TO), tomatine (TN), erythromycin (ERY) or ciprofloxacin (CIP) on the growth of the normal strain CF07-L and (C) of the SCV CF07-S. In (B), concentrations of 16 μg/ml of TO (n=3), 0.5 μg/ml of ERY (n=3) and 1.0 μg/ml of CIP (n=3) were used against CF07-L, whereas concentrations of 0.25 μg/ml of TO (n=4), 16 μg/ml of TN (n=3), 0.25 μg/ml of ERY (n=3) and 0.5 μg/ml of CIP (n=2) were used against CF07-S. The no drug control experiments are from 4 independent experiments.

Effect of tomatidine on the biosynthesis of macromolecules in HQNO-treated *S. aureus*. In order to get insight into the mechanism of action of tomatidine on SCVs, we performed macromolecular biosynthesis assays with the normal strain ATCC 29313 in the presence or absence of 20 μg HQNO/ml. We used HQNO-treated bacteria instead of SCVs because it was more reliable and allowed a reproducible production of elevated cell densities until the addition of HQNO. Fig 3A-3D show the results for the control antibiotics chloramphenicol, norfloxacin, rifampicin and vancomycin, which are well-known to target protein synthesis, DNA replication, RNA transcription and cell wall peptidoglycan synthesis, respectively. As expected, each of those antibiotics used at 4×MIC preferentially and significantly inhibited the incorporation of radiolabeled precursors into the targeted macromolecules although inhibition of other or all macromolecular biosynthesis also occur at concentrations >4×MIC, consistent with a collapse of cellular functions (data not shown). Tomatidine diluted in DMSO at a concentration of up to 125 μg/ml did not alter the synthesis of any macromolecule in ATCC 29213 in comparison to the DMSO-treated control (Fig. 3E). However, in the presence of 20 μg HQNO/ml, tomatidine decreased the biosynthesis of all macromolecules at concentrations equal or greater to 0.12 μg/ml (*i.e.*, the MIC recorded for all SCV strains tested) when compared to the HQNO-treated control (Fig.3F). In presence of HQNO, the inhibition of protein synthesis was significantly more affected by tomatidine ($P < 0.05$) than was the biosynthesis of all other macromolecules (Fig. 3F). This indicates that the primary cellular target of tomatidine could be the bacterial protein biosynthesis machinery.

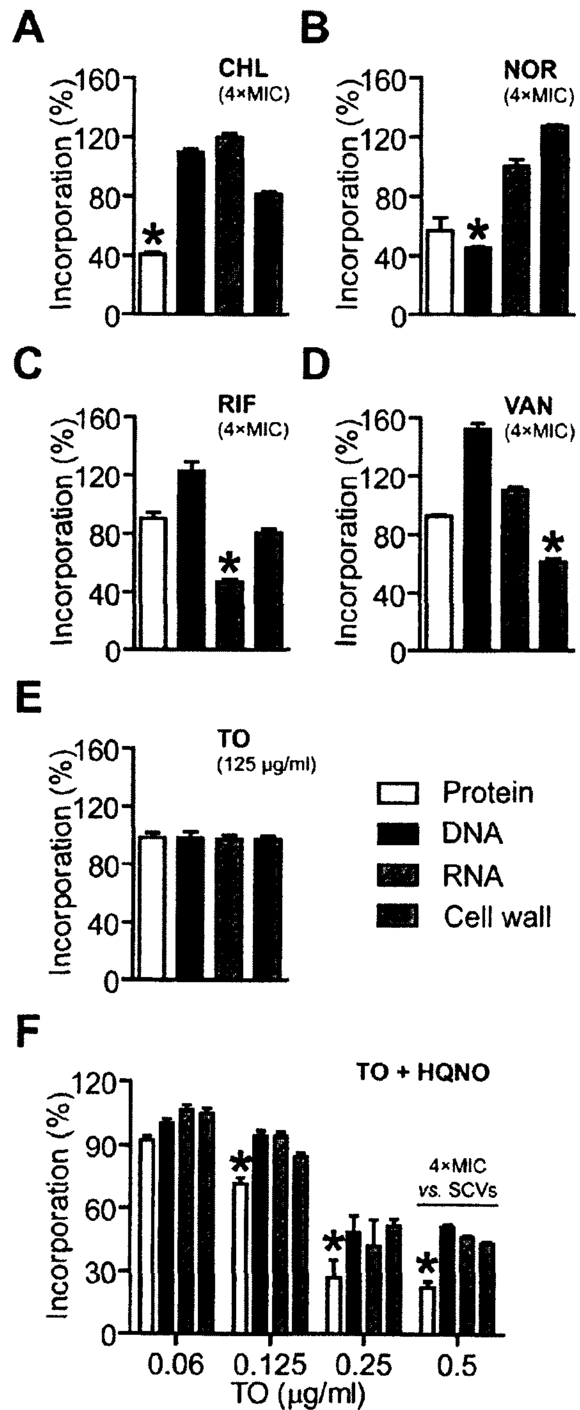
A
B
C
D
E
F
Incorporation (%)
CHL
(4×MIC)
NOR
(4×MIC)
RIF
(4×MIC)
VAN
(4×MIC)
TO
(125 µg/ml)
Protein
DNA
RNA
Cell wall
TO + HQNO
4×MIC
vs. SCVs
0.06
0.125
0.25
0.5
TO (µg/ml)

FIGURE 3. Effect of tomatidine on the biosynthesis of macromolecules in HQNO-treated *S. aureus*. (A-D) The effect of control antibiotics at approximately 4×MIC on the biosynthesis of proteins (chloramphenicol [CHL]; 64 μg/ml), DNA (Norfloxacin (Scott *et al.*); 4 μg/ml), RNA (Rifampicin (Beenken *et al.*); 0.06 μg/ml) and cell wall peptidoglycan synthesis (Vancomycin [VAN]; 4 μg/ml) was evaluated for strain ATCC 29213. (E) Effect of TO at 125 μg/ml on the biosynthesis of macromolecules in ATCC 29213. (F) Effect of different concentrations of TO on the biosynthesis of macromolecules in ATCC 29213 in the presence of HQNO at 20 μg /ml. Significant decreases of the biosynthesis of one macromolecule in comparison to all the three others are indicated (*, $P<0.05$, one-way ANOVA with Dunnett's post test for A,B,C,D and E and two-way ANOVA with a Bonferroni's post test for F). Results are from three independent experiments and are expressed as percentages of incorporation of radiolabeled-molecules by untreated (A-D), DMSO-treated (E) or HQNO-treated bacteria (F).

Tomatidine inhibits the replication of a clinical small-colony variant of *S. aureus* in polarized CF-like airway epithelial cells. SCVs are known to possess an increased ability to persist within non-phagocytic host cells (39) as confirmed by us in polarized CF-like airway epithelial cells (26). Fig. 4A shows that, although both strains caused similar level of infection at 24 h post-internalization, the intracellular load of SCV CF07-S 48 h post-internalization is clearly larger than that resulting from the normal strain CF07-L. These differences in cellular infection levels are likely to be explained by the ability of SCVs to persist and replicate within epithelial cells.

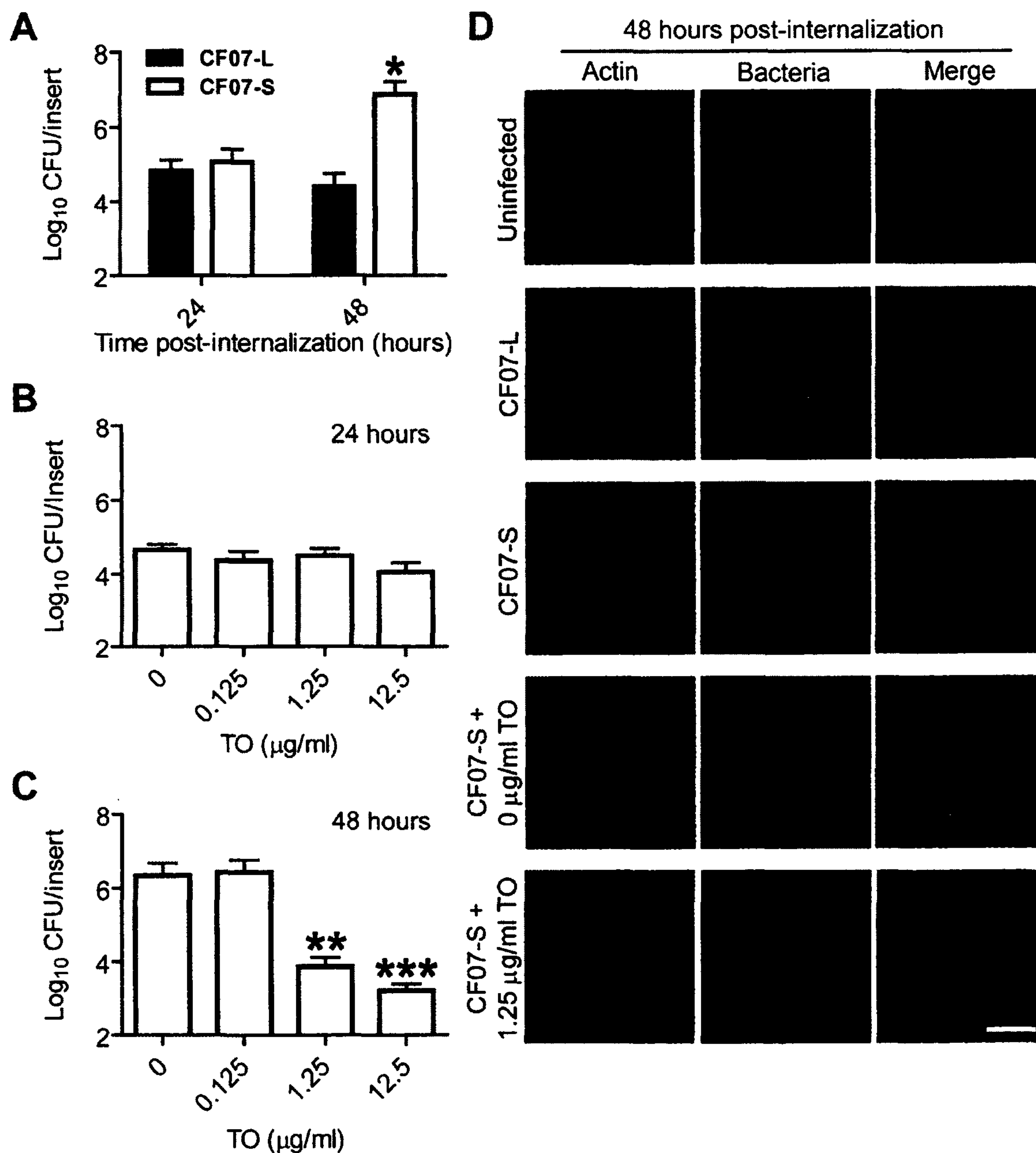

FIGURE 4. Tomatidine (TO) inhibits the replication of a clinical small-colony variant of *S. aureus* in polarized CF-like airway epithelial cells. (A) Infection levels of the polarized CF-like airway epithelial cells (the shCFTR cell line) with CF07-L and CF07-S 24 and 48 h post-internalization (from 2 to 3 independent experiments performed in duplicate). A significant difference between cells infected with CF07-L and CF07-S 48 h post-internalization is shown

(*, $P<0.05$; two-way ANOVA with the Bonferroni's post test). (B) Effect of different concentrations of TO on the intracellular load of CF07-S 24 h post-internalization. Data are from 2 independent experiments performed in duplicate. (C) Effect of different concentrations of TO on the intracellular load of CF07-S 48 h post-internalization. Data are from 3 independent experiments performed in duplicate. Significant differences in comparison to the control are shown (**, $P<0.01$; **, $P<0.001$; one-way ANOVA with a Dunnett's post test). Data are presented as means with standard deviations. (D) Fluorescence microscopy confirmed the capacity of tomatidine (1.25 µg/ml) to decrease the number of SCVs within cells in comparison to DMSO (0 µg/ml of tomatidine) 48 h post-internalization. *S. aureus* bacteria and cellular F-actin are stained red and green, respectively. Pictures are from stacked images acquired with a Zeiss microscope with ApoTome attachement. Scale bar is 100 µm.

We then evaluated the impact of tomatidine on the infection of epithelial cells by SCVs. We speculated that the bacteriostatic agent tomatidine could have a greater impact at 48 h post-internalization since replication of SCVs is most evident within these cells between 24 and 48 h. Indeed, Fig. 4B demonstrated that tomatidine did not have any effect on the infection of cells by the SCV CF07-S 24 h post-internalization. However, cells treated with 1.25 and 12.5 µg/ml of tomatidine contained significantly less SCVs than DMSO-treated cells 48 h post-internalization (Fig. 4C). Fig. 4D shows images obtained by fluorescence microscopy that confirmed the capacity of tomatidine to decrease the number of SCVs within cells 48 h post-internalization. Notheworthy, there was no differences in the number of nucleus per observed areas between cells treated for 48 h with DMSO and tomatidine at 12.5 µg /ml (data not shown), suggesting that the effect of tomatidine on the level of infection was not caused by a possible toxicity toward cells. These results demonstrated that tomatidine can significantly decrease the infection of polarized CF-like airway epithelial cells by SCVs.

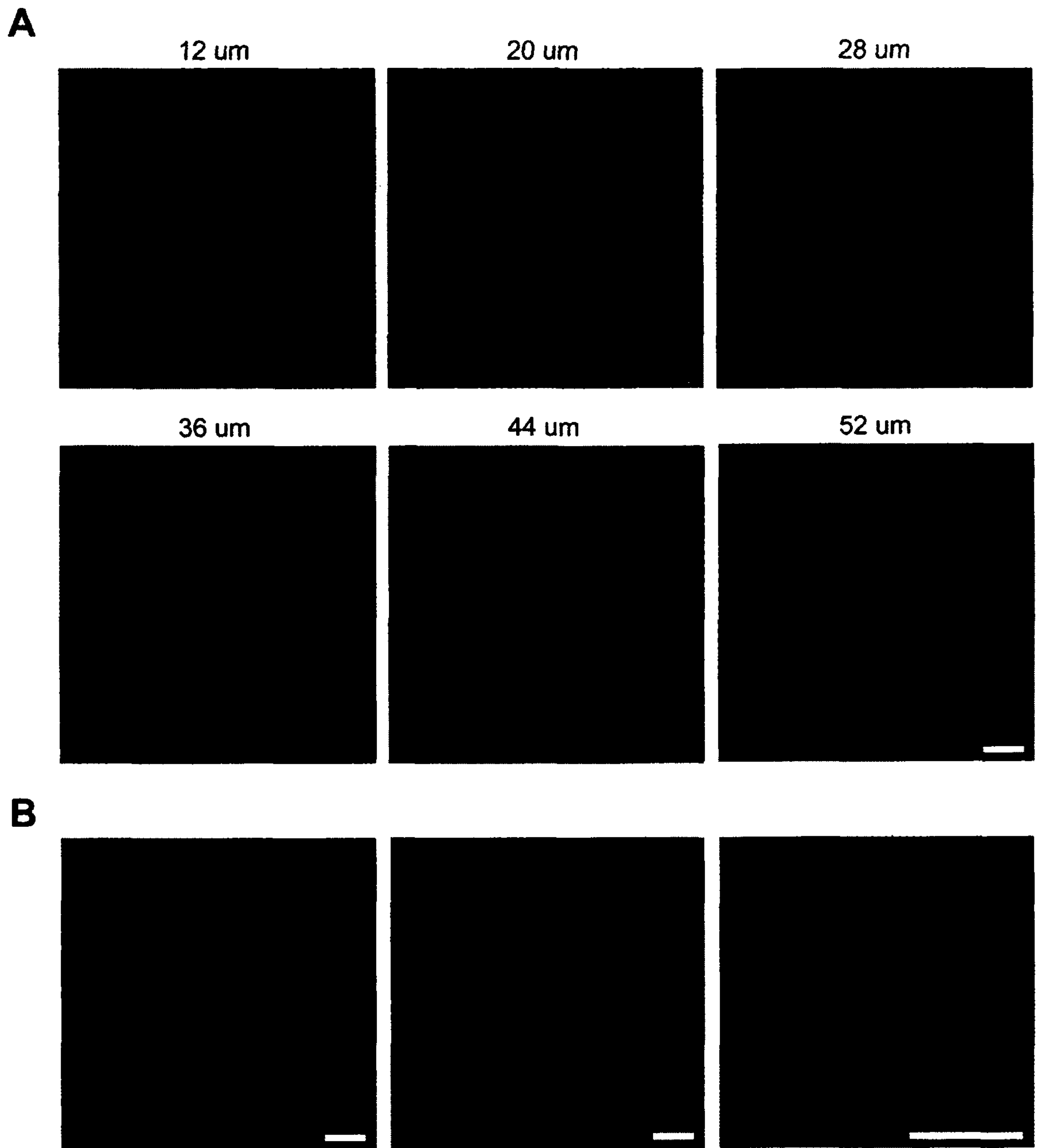

FIGURE 5. Confocal microscopy of polarized CF-like airway epithelial cells infected by the SCV CF07-S. (A) Z-series images of cells infected with CF07-S 48 h post-internalization. Distances from the bottom of the insert are indicated in microns (μm) above each picture. Scale bar is 100 μm. (B) Stacked images of selected layers obtained with confocal microscopy

showing *S. aureus* bacteria. Scale bars are 5 µm. *S. aureus* bacteria and cellular F-actin are stained red and green, respectively.

In order to confirm that the SCV CF07-S was still intracellular 48 h post-internalization, we performed confocal microscopy. Fig 5A shows an example of z-series images of shCFTR cells infected with CF07-S 48 h post-internalization. These images clearly suggest that almost all bacteria are inside the cell layers. Fig. 5B shows images acquired at higher magnifications and in which bacteria are clearly seen inside cells. Some antigenic materials were also detected at lower levels in the extracellular medium but are likely to represent dead bacteria or bacterial debris resulting from the action of the added lysostaphin. Overall, these results suggest that tomatidine inhibits the intracellular replication of SCVs in polarized CF-like airway epithelial cells.

Synergistic effect of tomatidine and gentamicin against a heterogeneous *S. aureus* population composed of both normal and SCV strains. We think that tomatidine could eventually be used in combination with classical antibiotics during therapies, especially that phenotypic switching between the normal and SCV phenotypes seems an integral part of *S. aureus* pathogenesis (46). We were therefore interested to evaluate if tomatidine could complete the antibacterial effect of the aminoglycoside gentamicin against a bacterial population composed of both normal and SCV strains (Table 2). Gentamicin at 4 µg/ml inhibits the growth of the normal strain CF07-L whereas tomatidine at 0.12 µg/ml does not. On the other hand, gentamicin at 4 µg /ml does not inhibit the growth of the SCV CF07-S while tomatidine at 0.12 µg /ml does. Hence, a combination of gentamicin at 4 µg /ml and tomatidine at 0.12 µg /ml inhibits the growth of a heterogeneous population composed of both the normal strain CF07-L and the SCV CF07-S whereas neither molecule alone can. These results support the hypothesis that tomatidine could be used in combination with classical

antibiotics and more particularly, in the context of chronic infections where the normal and SCV phenotypes co-exist.

TABLE 2. Combination of tomatidine and gentamicin inhibits the growth of a mixed population of normal and SCV strains co-isolated from a CF patient.

Strain[a]	GEN	TO	GEN+TO
CF07-L	-	+	-
CF07-S	+	-	-
CF07-L and CF07-S	+	+	-

[a]CF07-L is a normal strain whereas CF07-S is a SCV.

The precense (+) or the absence (-) of growth is indicated.

TO: tomatidine (0.12 ug/ml), GEN: gentamicin (4 ug/ml).

DISCUSSION

There are now numerous examples of plant secondary metabolites active against clinically-relevant pathogens and their use as antimicrobial agents, "antibiotic potentiators" or "virulence attenuators" for the control of infectious diseases is promising (15). Our preliminary work demonstrated that tomatidine has the ability to decrease the hemolytic activity and toxin production of normal *S. aureus* strains while also affecting the expression of virulence factors and biofilm formation in SCVs (6, 28). SCVs are often isolated from difficult-to-treat chronic infections (3, 30, 33) but no specific therapeutic approach against these variants had been considered to date. The present study demonstrated that tomatidine has in fact a specific

growth inhibitory activity against SCVs whereas it does not significantly affect the growth of normal strains. Tomatidine can thus be seen as both a virulence attenuator and an anti-SCV agent.

While the fundamental reason for the intrinsic resistance of normal strains to tomatidine is still unkown, our results suggest that the specific effect of tomatidine on SCVs is linked to their electron transport deficiency. Indeed, the metabolic complementation of either hemin- or menadione-dependent SCVs abolished the susceptibility of SCVs to tomatidine. Furthermore, we demonstrated that the electron transport inhibitor HQNO allows tomatidine to inhibit the growth of normal *S. aureus* strains. Interestingly, HQNO is an exoproduct of *Pseudomonas aeruginosa* recently associated with the formation of *S. aureus* SCVs (17, 29). This effect of *P. aeruginosa* on the phenotype of *S. aureus* could be especially relevant to cystic fibrosis (CF) as both organisms are commonly co-isolated from the airways of CF patients (16, 17). It is therefore possible that the presence of tomatidine could prevent the development of the SCV phenotype induced by interspecies interactions, thus reducing the likelihood of chronic *S. aureus* infections.

Although further investigations remain to be performed in order to completely understand the mechanism of action of tomatidine on *S. aureus*, our work suggests that tomatidine induces bacteriostasis in SCVs at a concentration as low as 0.12 µg/ml by inhibiting the biosynthesis of macromolecules, with a pronounced effect on protein synthesis. Other approaches such as ribosome binding experiments will need to be performed to verify this hypothesis. Based on our results, tomatidine may represent a new tool to combat SCV infections without exerting a selection pressure on *S. aureus* isolates of the normal phenotype. Moreover, reversion of SCVs to the normal phenotype, as observed during some of our experiments (data not shown), would provide resistance to the growth inhibitory activity of tomatidine but not necessarily to its anti-virulence effect. Furthermore, a combination therapy targeting both normal and SCV

phenotypes would presumably better control reversion and resistance. The toxicological and pharmacological properties of tomatidine have not yet been fully investigated and, consequently, the real potential of tomatidine as a therapeutic agent remains to be explored.

As mentioned before, SCVs are often isolated from chronic infections, more particularly from infections of the airways of cystic fibrosis patients. This propensity to cause chronic infections is thought to be related, at least in part, to the persistence of SCVs within non-phagocytic host cells (39). Antibiotic treatments are often ineffective against *S. aureus* infecting CF lungs and relapsing infections are often observed (13, 18). These relapsing infections are precisely thought to be caused by bacteria persisting inside host cells in the lungs (21). In fact, it is now thought that switching from the normal to the SCV phenotype and than back to the normal phenotype is an integral part of the pathogenesis of *S. aureus in vivo* and that novel therapeutic strategies targeting SCVs are needed to combat infections caused by bacterial species capable of generating such transitory variants (46). Our results show that the antimicrobial activity of tomatidine is effective against intracellular SCVs, which suggest that the clinical use of tomatidine may help to defeat difficult-to-treat and relapsing *S. aureus* infections caused by SCVs.

The use of tomatidine in combination with a classical antibiotic such as an aminoglycoside antibiotic is promising. Aminoglycosides are often used in the treatment of CF patients (22). However, it is known that SCVs are less susceptible to this class of antibiotics and are frequently isolated from the airways of CF patients (5, 19, 30). Moreover, it was shown by us and others that aminoglycosides can in fact induce the formation of SCVs (24, 27, 38). Thus, the combination of tomatidine with an aminoglycoside antibiotic in the treatment of CF patients should efficiently eradicate a population of *S. aureus* consisting of both the normal and SCV phenotypes. We are presently working at better characterizing the proprieties of

tomatidine but also at exploring derivatives to optimize their physico-chemical, antibacterial and anti-virulence properties.

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REFERENCES

1. **Alexander, E. H., and M. C. Hudson.** 2001. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. Appl. Microbiol. Biotechnol. **56:**361-6.
2. **Archer, G. L.** 1998. *Staphylococcus aureus*: a well-armed pathogen. Clin. Infect. Dis. **26:**1179-81.
3. **Atalla, H., C. Gyles, C. L. Jacob, H. Moisan, F. Malouin, and B. Mallard.** 2008. Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. Foodborne Pathog. Dis. **5:**785-99.
4. **Bednarek, P., and A. Osbourn.** 2009. Plant-microbe interactions: chemical diversity in plant defense. Science **324:**746-8.

5. **Besier, S., C. Smaczny, C. von Mallinckrodt, A. Krahl, H. Ackermann, V. Brade, and T. A. Wichelhaus.** 2007. Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in cystic fibrosis lung disease. J. Clin. Microbiol. **45:**168-72.
6. **Bouarab, K., El ordi, M., Gattuso, M., Moisan, H. and Malouin, F.** 2007. Plant stress response agents affect *Staphylococcus aureus* virulence genes. Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1483.
7. **Brouillette, E., A. Martinez, B. J. Boyll, N. E. Allen, and F. Malouin.** 2004. Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*. FEMS Immunol. Med. Microbiol. **41:**35-41.
8. **Bryan, L. E., and S. Kwan.** 1981. Aminoglycoside-resistant mutants of *Pseudomonas aeruginosa* deficient in cytochrome d, nitrite reductase, and aerobic transport. Antimicrob. Agents Chemother. **19:**958-64.
9. **Clinical and Laboratory Standards Institute (CLSI).** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved Standard.
10. **Cowan, M. M.** 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. **12:**564-82.
11. **Friedman, M.** 2002. Tomato glycoalkaloids: role in the plant and in the diet. J. Agric. Food Chem. **50:**5751-80.
12. **Galli, J., F. Ardito, L. Calo, L. Mancinelli, M. Imperiali, C. Parrilla, P. M. Picciotti, and G. Fadda.** 2007. Recurrent upper airway infections and bacterial biofilms. J. Laryngol. Otol. **121:**341-4.
13. **Goering, R. V., A. Bauernfeind, W. Lenz, and B. Przyklenk.** 1990. *Staphylococcus aureus* in patients with cystic fibrosis: an epidemiological analysis using a combination of traditional and molecular methods. Infection **18:**57-60.
14. **Goerke, C., and C. Wolz.** 2004. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. Int. J. Med. Microbiol. **294:**195-202.

15. **González-Lamothe, R., G. Mitchell, M. Gattuso, M. S. Diarra, F. Malouin, and K. Bouarab.** 2009. Plant antimicrobial agents and their effects on plant and human pathogens. Int. J. Mol. Sci. **10:**3400-19.

16. **Harrison, F.** 2007. Microbial ecology of the cystic fibrosis lung. Microbiology **153:**917-23.

17. **Hoffman, L. R., E. Déziel, D. A. D'Argenio, F. Lépine, J. Emerson, S. McNamara, R. L. Gibson, B. W. Ramsey, and S. I. Miller.** 2006. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U S A **103:**19890-5.

18. **Kahl, B., M. Herrmann, A. S. Everding, H. G. Koch, K. Becker, E. Harms, R. A. Proctor, and G. Peters.** 1998. Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. J. Infect. Dis. **177:**1023-9.

19. **Kahl, B. C., A. Duebbers, G. Lubritz, J. Haeberle, H. G. Koch, B. Ritzerfeld, M. Reilly, E. Harms, R. A. Proctor, M. Herrmann, and G. Peters.** 2003. Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. J. Clin. Microbiol. **41:**4424-7.

20. **Lightbown, J. W., and F. L. Jackson.** 1956. Inhibition of cytochrome systems of heart muscle and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-hydroxyquinoline N-oxides. Biochem. J. **63:**130-7.

21. **Lowy, F. D.** 2000. Is *Staphylococcus aureus* an intracellular pathogen? Trends Microbiol. **8:**341-3.

22. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194-222.

23. **Martin-Hernandez, A. M., M. Dufresne, V. Hugouvieux, R. Melton, and A. Osbourn.** 2000. Effects of targeted replacement of the tomatinase gene on the interaction of *Septoria lycopersici* with tomato plants. Mol. Plant. Microbe Interact. **13:**1301-11.

24. **Massey, R. C., A. Buckling, and S. J. Peacock.** 2001. Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. Curr. Biol. **11:**1810-4.

25. **Miller, M. H., S. C. Edberg, L. J. Mandel, C. F. Behar, and N. H. Steigbigel.** 1980. Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **18:**722-9.

26. **Mitchell, G., Bilodeau, G., Grondin, G., Cantin, A. and Malouin, F.** 2010. Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) increase *Staphylococcus aureus* intracellular infection of human pulmonary Cells. Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. D-1179.

27. **Mitchell, G., E. Brouillette, D. L. Séguin, A. E. Asselin, C. L. Jacob, and F. Malouin.** 2010. A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides. Microb. Pathog. **48:**18-27.

28. **Mitchell, G., Gattuso, M., Bouarab, K. and Malouin, F.** 2009. Tomatidine (TO) affects virulence regulators of prototypical *Staphylococcus aureus* (SA) and small colony variants (SCV) of cystic fibrosis (CF) patients. Abstr. 49th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1341.

29. **Mitchell, G., D. L. Séguin, A. E. Asselin, E. Déziel, A. M. Cantin, E. H. Frost, S. Michaud, and F. Malouin.** 2010. *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. BMC Microbiol. **10:**33.

30. **Moisan, H., E. Brouillette, C. L. Jacob, P. Langlois-Bégin, S. Michaud, and F. Malouin.** 2006. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J. Bacteriol. **188:**64-76.

31. **Osbourn, A. E.** 1996. Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack. Plant Cell **8:**1821-1831.

32. **Palmer, M. L., S. Y. Lee, P. J. Maniak, D. Carlson, S. C. Fahrenkrug, and S. M. O'Grady.** 2006. Protease-activated receptor regulation of Cl- secretion in Calu-3 cells requires prostaglandin release and CFTR activation. Am. J. Physiol. Cell Physiol. **290:**C1189-98.

33. **Proctor, R. A., C. von Eiff, B. C. Kahl, K. Becker, P. McNamara, M. Herrmann, and G. Peters.** 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat. Rev. Microbiol. **4:**295-305.

34. **Rios, J. L., and M. C. Recio.** 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. **100:**80-4.

35. **Roddick, J. G.** 1974. The steroidal glycoalkaloid α-tomatine. Phytochemistry **13:**9-25.

36. **Ruiz-Rubio, M., A. Perez-Espinosa, K. Lairini, T. Roldan-Arjona, A. Dipietro, and N. Anaya.** 2001. Metabolism of the tomato saponin α-tomatine by phytopathogenic fungi, p. 293-326. *In* A. Rahman (ed.), Studies in Natural Products Chemistry, vol. 25. Elsevier, Oxford.

37. **Sandrock, R. W., and H. D. Vanetten.** 1998. Fungal Sensitivity to and Enzymatic Degradation of the Phytoanticipin α-Tomatine. Phytopathology **88:**137-43.

38. **Schaaff, F., G. Bierbaum, N. Baumert, P. Bartmann, and H. G. Sahl.** 2003. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. Int. J. Med. Microbiol. **293:**427-35.

39. **Sendi, P., and R. A. Proctor.** 2009. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends Microbiol. **17:**54-8.

40. **Shah, P. M.** 2005. The need for new therapeutic agents: what is the pipeline? Clin. Microbiol. Infect. **11 Suppl 3:**36-42.

41. **Simons, V., J. P. Morrissey, M. Latijnhouwers, M. Csukai, A. Cleaver, C. Yarrow, and A. Osbourn.** 2006. Dual effects of plant steroidal alkaloids on *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **50:**2732-40.

42. **Singh, R., P. Ray, A. Das, and M. Sharma.** 2010. Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant. J. Med. Microbiol. **59:**521-7.

43. **Singh, R., P. Ray, A. Das, and M. Sharma.** 2009. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. J. Med. Microbiol. **58:**1067-73.

44. **Stewart, P. S.** 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. **292:**107-13.

45. **Talbot, G. H., J. Bradley, J. E. Edwards, Jr., D. Gilbert, M. Scheld, and J. G. Bartlett.** 2006. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin. Infect. Dis. **42:**657-68.

46. **Tuchscherr, L., E. Medina, M. Hussain, W. Völker, V. Heitmann, S. Niemann, D. Holzinger, J. Roth, R. A. Proctor, K. Becker, G. Peters, and B. Löffler.** 2011. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol. Med., **3**: 129-141.

CHAPITRE 7

Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant *Staphylococcus aureus* and prevents virulence gene expression

7.1. Introduction de l'article

Le but de cette étude était d'évaluer l'activité antibactérienne de la tomatidine en combinaison avec d'autres antibiotiques ainsi que d'évaluer l'effet de la tomatidine sur l'expression des facteurs de virulence chez *S. aureus*. J'ai découvert que la tomatidine et les aminoglycosides ont un effet synergique sur les souches normales de *S. aureus* au cours de mes expériences au laboratoire. J'ai par la suite participé à la supervision des étudiants Myriame Lafrance, Simon Boulanger et Isabelle Guay dans le cadre des essais de susceptibilité aux antibiotiques et de synergie. David Lalonde Séguin a mis au point et effectué les PCR afin de pouvoir détecter les différents gènes de résistance aux aminoglycosides. Mariza Gattuso a effectué les essais de PCR quantitative en temps réel. J'ai aussi effectué quelques essais de susceptibilité aux antibiotiques et de synergie ainsi que les essais d'hémolyse. J'ai fait l'analyse des résultats. Les Prs Éric Marsault et Kamal Bouarab sont des collaborateurs au projet. J'ai rédigé l'ébauche de l'article, effectué la conception des figures et participé à la correction du manuscrit jusqu'à sa publication. À noter que ce projet a mené au dépôt d'un brevet (voir Annexe 2, Résumé 3).

Référence de l'article :

Mitchell, G., Lafrance, M., Boulanger, S., Séguin, D.L., Guay, I., Gattuso, M., Marsault, E., Bouarab, K., and Malouin, F. (2012). Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant *Staphylococcus aureus* and prevents virulence gene expression. J. Antimicrob. Chemother. *67*, 559-568.

7.2. Résumé en français de l'article

OBJECTIFS: Cette étude a caractérisé les multiples activités biologiques du composé naturel tomatidine contre *Staphylococcus aureus*. Notamment, ce travail examine l'activité de la tomatidine en combinaison avec d'autres antibiotiques et l'influence de ce composé sur l'expression de certains facteurs de virulence chez *S. aureus*. MÉTHODES : L'effet de la tomatidine sur la susceptibilité de *S. aureus* à plusieurs classes d'antibiotiques a été déterminé par une procédure de microdilution en milieu liquide et un protocole de microdilution en damier (*chekerboard*) afin de mesurer les indices de concentrations inhibitrices fractionnelles et de révéler les interactions entre les composés. Des expériences de courbes de bactéridie avec des combinaisons aminoglycosides-tomatidine ont aussi été effectuées. L'activité hémolytique de plusieurs souches a été mesurée en présence de tomatidine sur des géloses sang. L'expression de gènes associés à la virulence a été évaluée chez la souche ATCC 29213 traitée à la tomatidine. RÉSULTATS : La tomatidine potentialisait spécifiquement l'effet inhibiteur des aminoglycosides mais pas celui d'autres classes d'antibiotiques. Cet effet de synergie était observé contre des souches de différentes origines cliniques (sang humain, voies respiratoires FK, ostéomyélite, peau humaine et mammite bovine) incluant des bactéries résistantes aux aminoglycosides possédant les gènes *aac(6')-aph(2'')*, *ant(4')-Ia* et/ou *aph(3')-IIIa*. Les courbes de bactéricidie pour les combinaisons aminoglycosides-tomatidine ont révélé une activité bactéricide forte. Même si la tomatidine ne possède pas d'activité inhibitrice de la croissance en soi contre les souches normales, elle inhibait l'activité hémolytique de plusieurs d'entre elles et, plus spécifiquement, interférait avec l'expression de plusieurs gènes normalement influencés par le système *agr*. CONCLUSIONS : Ces résultats montrent que la tomatidine potentialise l'action des aminoglycosides et agit en tant qu'agent antivirulence ciblant à la fois les souches de *S. aureus* susceptibles et résistantes aux antibiotiques.

7.3. L'article scientifique

Tomatidine acts in synergy with aminoglycoside antibiotics against multi resistant *Staphylococcus aureus* and prevents virulence gene expression

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Running title: Tomatidine is an antibiotic potentiator and an anti-virulence agent.

Keywords: Aminoglycoside-resistant staphylococci, MRSA, cystic fibrosis, steroidal alkaloid, antibiotic synergy.

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SYNOPSIS

Objectives: This study characterized the multiple biological activities of the natural compound tomatidine against *Staphylococcus aureus*. Notably, this work examined the antibacterial activity of tomatidine in combination with other antibiotics and the influence of this compound on the expression of virulence factors in *S. aureus*. **Methods:** The effect of tomatidine on the susceptibility of *S. aureus* to several antibiotic classes was determined by a broth microdilution procedure and a checkerboard protocol to measure fractional inhibitory concentration indices and to reveal drug interactions. Time-kill experiments for aminoglycoside-tomatidine combinations were also performed. The hemolytic ability of several strains in presence of tomatidine was measured on blood agar plates and the expression of virulence-associated genes in strain ATCC 29213 treated with tomatidine was monitored by quantitative PCR. **Results:** Tomatidine specifically potentiated the inhibitory effect of aminoglycosides but not of other classes of drugs. This potentiating effect was observed against strains of different clinical origins (human blood, cystic fibrosis airways, osteomyelitis, skin tissues and bovine mastitis) including aminoglycoside-resistant bacteria possessing the *aac(6')-aph(2'')*, *ant(4')-Ia* and *aph(3')-IIIa* genes. The killing kinetics for the combination of aminoglycosides with tomatidine revealed strong bactericidal activity. Although tomatidine did not possess growth-inhibitory activity of its own against prototypical *S. aureus*, it inhibited the hemolytic activity of several strains and more specifically, blocked the expression of several genes normally influenced by the *agr* system. **Conclusions:** These results show that tomatidine is an aminoglycoside potentiator that also acts as an anti-virulence agent targeting both antibiotic-susceptible and antibiotic-resistant *S. aureus*.

INTRODUCTION

Antibiotic resistance now represents an overwhelming problem in human and veterinary medicine since pathogens have developed mechanisms to circumvent almost all antibiotics of the current arsenal.[1, 2] Staphylococcal infections are often refractory to antibiotic treatments[3] and cause both life-threatening and chronic diseases affecting several hosts and body sites.[4-6] *Staphylococcus aureus* is a significant pathogen causing difficult-to-treat infections[2] and is commonly recovered from cystic fibrosis (CF) patients.[7-9] Antibiotic therapy usually leads to better clinical prognostics in CF.[10, 11] However, the emergence of antibiotic-resistant bacteria is a major problem for patients afflicted by this disease and the prevalence of methicillin-resistant *S. aureus* (MRSA) is currently increasing among CF patients.[11] This is alarming considering that MRSA are most often resistant to multiple antibiotic classes[12] and are associated with declines of lung function and worse survival in CF patients.[13, 14] MRSA are associated with hospital- and community-acquired infections (HA- and CA-MRSA)[14] but are also found in livestock (LA-MRSA) and companion animals and transmission from colonized animals to humans has been reported.[15, 16]

The ability of *S. aureus* to cause multiple diseases is related to its collection of virulence factors, the expression of which is controlled by a variety of global regulators that allow diverse strategies for pathogenesis. Such strategies include bacterial adhesion to extracellular matrix components, the formation of biofilms, immunomodulation, the destruction of host tissues and the invasion of host cells.[4] The quorum-sensing dependent *agr* system is known to be a major regulator of staphylococcal virulence.[17, 18] Once activated, the *agr* system leads to down-regulation of several cell-surface proteins such as adhesins and to up-regulation of several exotoxins and hydrolytic enzymes. It was shown that the *agr* system greatly influences the outcome of *S. aureus* infection in several experimental models.[18] Targeting virulence would thus be an interesting new avenue for drug development.

Plant products providing antibiotic-like activities against a wide variety of pathogenic bacteria may represent new chemical scaffolds for the development of antimicrobials.[19] These plant products may directly inhibit or kill pathogenic bacteria, but some may alternatively increase the antimicrobial activity of already known antibiotics ("antibiotic potentiators") or alter the virulence and/or the adaptation of pathogen toward the host ("virulence attenuators").[19] The plant product tomatidine and other closely related steroidal alkaloids do not have any clinically significant growth-inhibitory activity against prototypical *S. aureus*.[20, 21] We recently discovered serendipitously that tomatidine possess an important and specific bacteriostatic activity against respiratory-deficient *S. aureus* small-colony variants (SCVs).[20] The clinical importance of SCVs is increasingly recognized as their ability to persist in non-professional phagocytes and their association with chronic infections is now well established.[22] The development of antibiotics able to target both extra and intracellular SCVs such as tomatidine[20] could represent interesting alternatives for clinicians. However, SCVs are rarely found alone during infections and are often co-isolated with strains harboring the normal phenotype.[22, 23] In fact, it has been proposed that phenotypic switching is intrinsic to *S. aureus* pathogenesis.[24] Consequently, this study examined the interaction of tomatidine with antibiotics of several classes in order to reveal useful combination therapies targeting both the normal and SCV phenotypes of *S. aureus*. Furthermore, although tomatidine alone does not significantly affect the growth of prototypical *S. aureus*, we evaluated its influence on virulence gene expression to reveal additional beneficial effects of this natural product.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A variety of *S. aureus* clinical strains of human and veterinary origins were used in this study. MRSA Sa220c and Sa228c were isolated from human skin and soft tissue infections at the Centre Hospitalier Universitaire de Sherbrooke (CHUS). Several *S. aureus* isolates (CF07-L,

CF1A-L, CF2A-L, CF4B-L, CF6B-L, CF7A-L, CF8E-L, CF9A-L, CF35A-L) were isolated from the airways of CF patients by the clinic of the CHUS. The strains were collected following a consent protocol approved by the ethics review board of the CHUS (protocol 06-158-R4). MA078038 is a human blood isolate (CMRSA-10/USA300) from the Laboratoire de Santé Publique du Québec (LSPQ). Strain SHY97-3906 is a bovine mastitis isolate.[25] NewbouldΔ*sigB* was generated from Newbould (ATCC 29740, another bovine mastitis isolate) by disrupting the *sigB* gene with the *ermA* cassette by homologous recombination.[26] Additional *S. aureus* reference strains were also used such as ATCC 29213, Newman, MRSA COL, MRSA ATCC 43300, MRSA ATCC BAA-41, MRSA N315, and MRSA Mu50, which is also a vancomycin intermediate resistant *S. aureus* (VISA). Strain 8325-4 is characterized by lower SigB activity resulting from a "natural" deletion in the *rsbU* locus.[27] SH1000 is isogenic to strain 8325-4, but with a functional *rsbU* allele.[27] Except where otherwise stated, bacteria were grown in cation-adjusted Mueller-Hinton broth (CAMHB) (BD, Mississauga, ON, Canada).

Chemical reagents and antibiotics

Tomatidine hydrochloride, oxacillin, erythromycin, ciprofloxacin, tetracycline, vancomycin, gentamicin, kanamycin, tobramycin, amikacin and streptomycin were from Sigma (Oakville, ON, Canada). Tomatine, the lycotetraose-substituted derivative of tomatidine, was from ICN biomedicals (Irvine, CA, USA). Tomatidine and tomatine were solubilized at 2 g/L in DMSO. Tomatidine was warmed at 70°C during the solubilisation process. Oxacillin, ciprofloxacin, tetracycline, vancomycin, gentamicin, kanamycin, tobramycin, amikacin and streptomycin were solubilized in water at 10 g/L. NaOH 2.5 N was used during the solubilisation of ciprofloxacin. Erythromycin was solubilised at 10 g/L in 1:1 water:ethanol. The chemical structures of tomatidine and tomatine are shown in Figure 1.

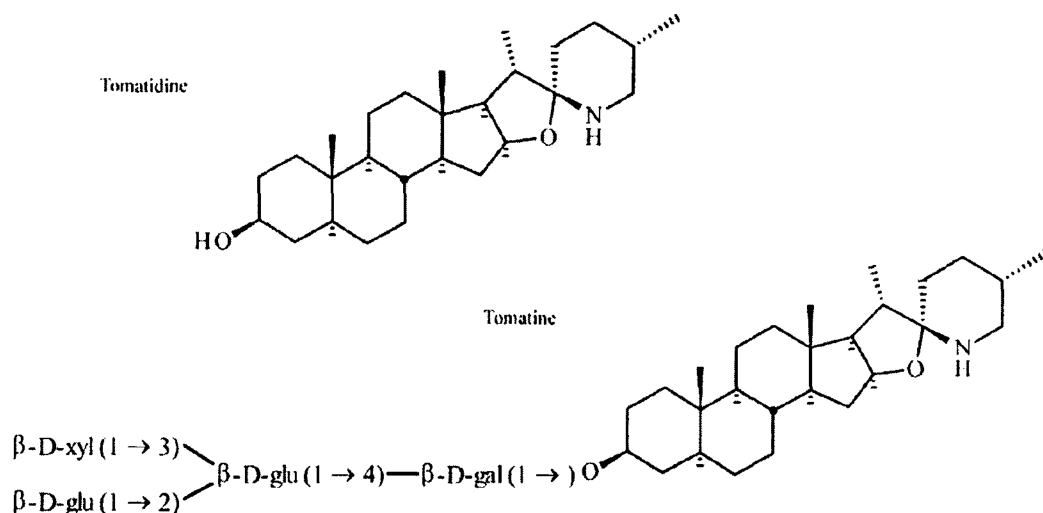

FIGURE 1. Chemical structures of tomatidine (TO) and tomatine (TN).

Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by a broth microdilution technique, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[28] *S. aureus* ATCC 29213 was used as a comparator strain in all MIC tests. Resistance phenotypes were attributed using the following susceptibility breakpoints: oxacillin 4 mg/L, erythromycin 8 mg/L, ciprofloxacin 4 mg/L, tetracycline 16 mg/L, vancomycin 16 mg/L, gentamicin 16 mg/L, kanamycin 64 mg/L and tobramycin 16 mg/L. Intermediate resistance to gentamicin and vancomycin was defined by concentrations of 8 and 4-8 mg/L, respectively. The concentration of tomatidine chosen for antibiotic combinations in MIC assays was 4-8 mg/L, which was well above the minimal tomatidine concentration showing a potentiating effect in synergy studies (see the Results section).

Synergy studies

A checkerboard protocol[29] was conducted by a broth microdilution method similar to that used for standard MIC determinations in order to evaluate the effect of varying concentrations of tomatidine on the activity of aminoglycoside antibiotics against *S. aureus* strain ATCC 29213. The fractional inhibitory concentration (FIC)[29] indices were calculated as follows: FIC index = FICA + FICB = A/MICA+ B/MICB, where A and B are the MICs of compounds A and B in combination, MICA and MICB are the MICs of compound A and compound B alone and FICA and FICB are the FICs of compound A and of compound B. Indifference for drug interactions or an additive effect is demonstrated if the FIC index is >0.5-4 and synergy if the FIC index is ≤ 0.5, whereas an antagonistic effect is represented by an FIC index of >4.[30]

Time-kill experiments

Time-kill kinetics were followed in order to determine whether the effect of compounds alone or in combination was bacteriostatic or bactericidal. Bacteria were inoculated at $\sim 10^5$-10^6 cfu/mL in 100 mL CAMHB and grown at 35°C with shaking in the absence or presence of antibiotics at the following concentrations (as specified in figure legends): tomatidine and tomatine, 8 mg/L; ciprofloxacin and erythromycin, 1 mg/L; gentamicin, 0.12-4 mg/L. At several timepoints, bacteria were sampled, serially diluted and plated on tryptic soy agar (TSA) for cfu determinations.

Aminoglycoside resistance determinants

The aminoglycoside-resistant strains used in this study were screened by PCR for the presence of genes encoding aminoglycoside-modifying enzymes. PCR detection of the *aac(6')-aph(2'')* (351bp), *ant(4')-Ia* (172bp) and *aph(3')-IIIa* (268bp) amplicons was done by using the following primer sequences: *aac(6')-aph(2''),* FWD 5'-ACAGAGCCTTGGGAAGATGAAGT-3', REV 5'-

GCCCTCGTGTAATTCATGTTCTGGC-3'; *ant(4')-Ia,* FWD 5'-CTGCTAAATCGGTAGAAGC-3', REV 5'-CAGACCAATCAACATGGCACC -3'; *aph(3')-IIIa,* FWD 5'-CTGATCGAAAAATACCGCTGC-3', REV 5'-TCATACTCTTCCGAGCAAAGG-3'. Primers for the amplification of *aac(6')-aph(2'')* were designed using the *aac(6')-aph(2'')* DNA sequence of strain Mu50. Primers for the amplification of *ant(4')-Ia* and *aph(3')-IIIa* were from Schmitz *et al.*[31] Samples were denatured at 94°C for 10 min followed by 35 amplification cycles using the following parameters: 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A final extension cycle of 72°C for 10 min was used. After amplification, PCR products were resolved using 1.5% (w/v) agarose gels.

Hemolysis

Bacterial suspensions of 0.5 McFarland were prepared for each strain and were spotted (2 µL) on Mueller-Hinton agar (BD, Mississauga, ON, Canada) with 5% horse blood supplemented or not with 8 mg/L of tomatidine or tomatine. Hemolysis was observed after 24 h of incubation at 35°C followed by an overnight incubation at 4°C.

Quantitative PCR

In order to evaluate the effect of tomatidine or tomatine on *S. aureus* gene expression, overnight cultures were used to inoculate CAMHB at an $A_{600\ nm}$ of 0.1. Bacteria were then grown until an $A_{600\ nm}$ of 0.6 and exposed to tomatidine or tomatine for 3 h. RNA extraction, cDNA synthesis and qPCR were performed as previously described[26, 32] using the primers described in Table 1. The relative expression ratios were calculated by using the cycle threshold (C_t) of the untreated culture (no-drug control) and that of the housekeeping *gyrA* gene (*n*-fold expression = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t$ (treated culture) / ΔC_t (untreated culture) and ΔC_t represents the difference between the C_t of the gene studied and the C_t of *gyrA*).

Statistical analysis

Statistical analyses were carried out with the GraphPad Prism Software (v.5.00). For qPCR results, analyses were performed on $\Delta\Delta C_t$. Statistical tests used for the analysis of each experiment are specified in the Results section and the Figure legends.

TABLE 1. Quantitative PCR Primers used in this study.

ORF SACOL	Gene	Description	Primer	Sequence
6	*gyrA*	DNA gyrase, A subunit	6-RT-FWD	CATTGCCAGATGTTCGTGAC
			6-RT-REV	CACCAACGATACGTGCTGAT
78	*plc*	Phospholipase	78-RT-FWD	GGAGGCAGCGATTTAATAG
			78-RT-RV	AGCCAACCCGTTCTAGCCTTT
860	*nuc*	Thermonuclease	860-RT-FWD	TCGCTTGCTATGATTGTGGTAGCC
			860-RT-REV	TACAGGCGTATTCGGTTTCACCGT
1173	*hla*	α-Hemolysin	1173-RT-FWD	AATGAATCCTGTCGCTAATGCCGC
			1173-RT-REV	CTGAAGGCCAGGCTAAACCACTTT
1867	*splC*	Serine protease	1867-RT-FWD	AACGGTGACAAAGGAAATGG
			1867-RT-REV	CTTTTGGTCCACGTTCGACT
2022	*hld*	δ-Hemolysin, RNA III	2022-RT-FWD	TAATTAAGGAAGGAGTGATTTCAATG
			2022-RT-REV	TTTTTAGTGAATTTGTTCACTGTGTC
2694	*geh*	Lipase	2694-RT-FWD	GTTGGCTCAATGGGGTCTAA
			2694-RT-REV	CTCACGCGTCAGATCGTAAA

ORF SACOL, open reading frame number in *S. aureus* COL.

RT-FWD, real-time PCR forward primer; RT-REV, real-time PCR reverse primer.

RESULTS

Tomatidine potentiates the action of aminoglycoside antibiotics against *S. aureus*. We showed previously that tomatidine had potent inhibitory activity against *S. aureus* SCVs but

that this natural product had no clinically significant MIC against normal strains of *S. aureus*.[20] In order to further characterize the effect of tomatidine on *S. aureus* and to better understand its therapeutic potential, we evaluated the activity of tomatidine in combination with several classes of antibiotics. We found that tomatidine increased the susceptibility (*e.i.* decreased the MIC) of ATCC29213 to the aminoglycoside antibiotics gentamicin (8-32 fold), kanamycin (4-8 fold), tobramycin (4-8 fold), amikacin (4 fold) and streptomycin (4-8 fold), whereas it did not alter that of other classes of antibiotics (oxacillin [β-lactams], erythromycin [macrolides], ciprofloxacin [fluoroquinolones], tetracycline [tetracyclines], vancomycin [glycopeptides]) (Table 2). The specificity of tomatidine as an aminoglycoside potentiator was also demonstrated by the use of the structurally related tomatine (Figure 1), which did not influence the susceptibility of ATCC 29213 to aminoglycosides or any other classes of antibiotics (data not shown).

TABLE 2. Effect of tomatidine on the susceptibility of *S. aureus* ATCC 29213 to several antibiotics.

Antibiotic	MIC (mg/L)			FIC index[b]
	-TO	+TO	FOLD[a]	
Gentamicin	0.5-1	0.03-0.06	8-32	≤ 0.116
Kanamycin	2-4	0.5	4-8	≤ 0.193
Tobramycin	0.25-0.5	0.06	4-8	≤ 0.133
Amikacin	2	0.5	4	≤ 0.133
Streptomycin	4-8	1	4-8	≤ 0.199
Oxacillin	0.25	0.25	1	ND
Erythromycin	0.5	0.5	1	ND
Ciprofloxacin	0.5	0.5	1	ND
Tetracycline	0.25-0.5	0.25-0.5	1	ND
Vancomycin	1	1	1	ND

[a]Ratios expressed as fold differences between MICs without (-) and with (+) TO were determined for each independent experiment.

[b]FIC indexes were determined by the checkerboard protocol.

TO: tomatidine, ND: not determined.

The activity of tomatidine in combination with gentamicin and/or tobramycin was also studied against other Gram-positive bacteria. Tomatidine potentiated the activity of gentamicin and tobamycin against *Staphylococcus epidermidis* (2-8 fold), *Staphylococcus haemolyticus* (4-16 fold), *Staphylococcus saprophyticus* (1-8 fold) and *Staphylococcus hominis* (2-4 fold), but not against *Enterococcus faecalis* and *Enterococcus faecium* (data not shown). For evaluation against Gram-negative bacteria, the activity of tomatidine in combination with tobramycin was tested against *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 with no effect. Other *E. coli* strains tested that also showed no increase in susceptibility to the tomatidine-aminoglycoside combination included a strain highly permeable to large antibiotic molecules like erythromycin and vancomycin (*imp4213*),[33] a strain that lacks one of the major efflux pump systems (*acrAB::aph(3')*)[34] and a rough strain (*E. coli* J5) lacking the long lipopolysaccharide (LPS) chains that prevent lipophilic molecules from interacting with bacterial membranes.[35]

The effect of tomatidine on the susceptibility of ATCC 29213 to several aminoglycoside antibiotics (gentamicin, kanamycin, tobramycin, amikacin and streptomycin) was confirmed using the checkerboard method. Results showed that tomatidine markedly increased the susceptibility of ATCC 29213 to all aminoglycoside antibiotics tested at a concentration as low as 0.12 mg/L (data not shown). At this concentration and above (tested up to 8 mg/L), a reduction of 4 to 32 fold in the MICs of aminoglycosides was observed. To approximate the FIC indices, we considered an MIC of 32 mg/L for tomatidine against ATCC 29213. This MIC was knowingly overestimated because there is still no complete growth inhibition of normal *S. aureus* strains in the presence of tomatidine at concentrations as high as 128 mg/L.[19] The approximate FIC values characterizing the interaction of tomatidine with aminoglycoside antibiotics are reported in Table 2 and demonstrate strong synergy between the antimicrobial activities of these molecules (FIC < 0.5).

TABLE 3. Effect of tomatidine on the susceptibility of *S. aureus* strains of diverse origins and antibiotic suceptibility profiles to aminoglycosides

Strains	Details[a]	Aminoglycoside modifying enzyme-encoding	MIC (mg/L)[b]														
			OXA	ERY	CIP	TET	VAN	GEN	GEN +TO	FOLD[c]	KAN	KAN + TO	FOLD[c]	TOB	TOB + TO	FOLD[c]
Strains suceptible to GEN, KAN and TOB																	
8325-4	-	-	0.12	0.25-0.5	0.25-0.5	0.25	1	0.12-0.25	0.03-0.06	4	2-8	0.5-4	2-4	0.12-0.25	0.03-0.06	4
COL	MRSA	-	>64 (R)	0.5	0.5	1-2	2	0.5-1	0.06	8-16	4	0.5	8	0.5-1	0.06	8-16
Newman	OS	-	0.5-1	0.5	0.25	0.25-0.5	1	0.5-1	0.06	8-16	4	0.5	8	0.25-0.5	0.06-0.12	2-8
Newbould	BM	-	0.12	0.5	0.25	0.25-0.5	0.5-1	0.5-1	0.06	8-16	4	0.5	8	0.5-1	0.06-0.12	8
SHY97-3906	BM	-	0.12-0.25	0.12-0.25	0.25	0.25-0.5	0.5-1	0.25	0.03-0.06	4-8	2	0.5	4	0.25	0.03	8
Sa220c	SSTI, MRSA	-	16-32 (R)	0.5-1	32 (R)	0.5	1	0.5	0.06-0.12	4-8	4	0.5	8	0.5-1	0.06-0.12	4-16
CF07-L	CF	-	0.12-0.25	0.5	0.5	0.5	1-2	0.5-1	0.06-0.25	4-8	4	0.5-1	4-8	0.5-1	0.06-0.12	4-8
CF1A-L	CF	-	0.25-0.5	0.25-0.5	0.5-1	0.25	1	0.5-1	0.12	4-8	4	0.5	8	1	0.06-0.25	4-16
CF2A-L	CF	-	0.25	0.5	0.25	2	1	1	0.12-0.25	4-8	4	0.5	8	1	0.06-0.12	8-16
CF4B-L	CF	-	0.5	0.5	0.25-0.5	0.25	1	0.5	0.06-0.12	4-8	4	0.5	4-8	0.5-1	0.06-0.12	4-16
CF8E-L	CF	-	0.125	0.5	1	0.25-0.5	1-2	1	0.25-0.5	2-4	16	2-8	2-4	1	0.25	4
CF35A-L	CF, MRSA	-	>64 (R)	>64 (R)	>64 (R)	0.5	1	0.5-1	0.06-0.12	8	4	1	4	0.5-1	0.06	8-16
Strains resistant to GEN, KAN and/or TOB																	
ATCC 43300	MRSA	ND[d]	16-32 (R)	>64 (R)	0.5	0.5	0.5-1	64-128 (R)	16-32 (R)	4	512-1024 (R)	256 (R)	2-4	512-1024 (R)	128 (R)	4-8
ATCC BAA-41	MRSA	*ant(4')-Ia*	>64 (R)	>64 (R)	>64 (R)	0.5	1	0.5	0.12-0.25	2-4	256-512 (R)	64 (R)	4-8	512 (R)	128 (R)	4
N315	MRSA	*ant(4')-Ia*	8 (R)	>64 (R)	0.25	0.5	0.5	1	0.12	8	256 (R)	64 (R)	4	512 (R)	128-256 (R)	2-4
Mu50	MRSA, VISA	*aac(6')-aph(2'')* *ant(4')-Ia*	>64 (R)	>64 (R)	32-64 (R)	>16 (R)	4 (I)	128 (R)	16 (R)	8	>1024 (R)	256-512 (R)	>2-4	1024 (R)	128 (R)	8
MA078038	BI, MRSA	*aph(3')-IIIa*	64 (R)	64 (R)	16 (R)	0.5	0.5-1	0.25-0.5	0.06	4-8	>1024 (R)	1024 (R)	>1	0.5	0.06-0.12	4-8
Sa228c	SSTI, MRSA	*aac(6')-aph(2'')* *ant(4')-Ia*	>64 (R)	>64 (R)	>64 (R)	>16 (R)	1	64-128 (R)	8-16 (I)	8	512-1024 (R)	128-256 (R)	2-8	1024 (R)	128-256 (R)	4-8
CF6B-L	CF	*aac(6')-aph(2'')*	0.25-1	0.5-1	>64 (R)	0.25	0.5-1	128-256 (R)	16-32 (R)	4-8	1024 (R)	128-256 (R)	4-8	256 (R)	16-64 (R)	4-16
CF7A-L	CF, MRSA	*ant(4')-Ia*	>64 (R)	>64 (R)	>64 (R)	0.25-0.5	1	0.5-1	0.12-0.25	2-8	256-512 (R)	64-256 (R)	2-4	1024 (R)	128-512 (R)	2-8
CF9A-L	CF, MRSA	*ant(4')-Ia*	>64 (R)	>64 (R)	>64 (R)	0.25-0.5	1	0.5-1	0.06-0.12	4-8	256 (R)	128-256 (R)	1-2	512-1024 (R)	64-256 (R)	4-8

[a]MRSA: methicillin-resistant *S. aureus* , VISA: vancomycin intermediate resistant *S. aureus* . BI: blood, BM: bovine mastitis, CF: cystic fibrosis airways, OS: osteomyelitis, SSTI: skin and soft tissue infection.

[b]OXA: oxacillin, ERY: erythromycin, CIP: ciprofloxacin, TET: tetracycline, VAN: vancomycin, GEN: gentamicin, TOB: tobramycin, KAN: kanamycin, TO: tomatidine. Intermediate resistance (I) and resistance (R) are indicated.

[d]Ratios expressed as fold differences between the aminoglycoside MIC without and with the presence of tomatidine were determined for each independent experiment and show the increase in *S. aureus* susceptibility.

[d]ND: not determined.

Tomatidine specifically potentiates the action of aminoglycosides against *S. aureus* strains of various clinical origins and antibiotic susceptibility profiles. The antibacterial effect of tomatidine in combination with aminoglycosides was next evaluated against a group of *S. aureus* strains of diverse origins. These strains were isolated from a variety of diseases and body sites, such as bovine mastitis, human osteomyelitis, blood, skin and soft tissue infections and from the airways of CF patients, and are shown in Table 3. Table 3 also presents the MICs of oxacillin, erythromycin, ciprofloxacin, tetracycline, vancomycin and three aminoglycosides (gentamicin, kanamycin, tobramycin) against these *S. aureus* strains. It is noteworthy that several of these strains were resistant to multiple classes of antibiotics and/or are MRSA. Importantly, Table 3 shows that tomatidine increased the susceptibility of all the studied strains to aminoglycoside antibiotics. More precisely, the presence of tomatidine could increase the susceptibility of aminoglycoside-susceptible *S. aureus* strains to gentamicin, kanamycin or tobramycin by 2 to 16 fold.

Interestingly, the potentiating effect of tomatidine on the activity of aminoglycoside antibiotics was also detectable against aminoglycoside-resistant *S. aureus* strains (Table 3). Strains resistant to gentamicin, kanamycin and/or tobramycin were screened for genetic determinants encoding aminoglycoside-modifying enzymes (AMEs), which represent the main mechanism of aminoglycoside resistance.[31, 36, 37] The detected AME genes suited the expected aminoglycoside-suceptibility pattern for each strain. The presence of *aac(6')-aph(2'')* correlated with resistance to gentamicin, kanamycin and tobramycin, that of *ant(4')-Ia*, with resistance to kanamycin and tobramycin and that of *aph(3')-IIIa* with resistance to kanamycin (Table 3). Interestingly, as for the aminoglycoside-susceptible strains, the activity of aminoglycosides against strains carrying AME genes was also generally increased by 2-16 fold in the presence of tomatidine although in this case MICs did not drop below the resistance breakpoints (Table 3). Notably, although we have previously shown that tomatidine possesses a strong antibacterial effect of its own against *S. aureus* SCVs (MIC of 0.12 mg/L),[20] we did not observe any synergy with aminoglycosides against such variants, which are already highly

susceptible to tomatidine but less susceptible to aminoglycosides with gentamicin MIC of 4-8 mg/L (data not shown).

The combination of tomatidine with aminoglycoside antibiotics is bactericidal. Time-kill experiments were performed in order to determine whether the combination of tomatidine with aminoglycoside antibiotics is bacteriostatic or bactericidal against *S. aureus*. Figure 2(a) shows the effect of tomatidine and control antibiotics (erythromycin and ciprofloxacin at 2×MIC) on the viability of *S. aureus* ATCC 29213 as a function of time. As expected, tomatidine did not affect the growth or viability of *S. aureus* (MIC >128 mg/L), while erythromycin was bacteriostatic and ciprofloxacin was bactericidal (>3 $\log_{10}$ reduction in cfu). Figure 2(b) shows that gentamicin alone at ~1-2×MIC (gentamicin MIC was 0.5-1 mg/L, Table 3) was also bactericidal against *S. aureus,*[38] although, as anticipated for aminoglycosides, regrowth was observed within 24 h.[39, 40] However, Figure 2(b) also shows that re-growth of bacteria was markedly reduced when tomatidine was combined with gentamicin at 1 mg/L and shows that this combination produced a significant synergy and a bactericidal effect that was sustained for 24 h. Interestingly, the combination of a subinhibitory concentration of gentamicin (0.12 mg/L, *i.e.*, about 1/4 to 1/8 of the MIC) with tomatidine resulted in a bactericidal effect (Figure 2c) that was quicker than that seen with ciprofloxacin at 2×MIC (Figure 2a), although, as for the aminoglycoside alone at ~1-2×MIC, regrowth was observed within 24 h. Again, this potentiating effect was not observed with tomatine, confirming the specificity of tomatidine. Note that similar results were obtained for tobramycin at 0.12 and 1 mg/L in combination with tomatidine (data not shown).

In order to examine the possibility that tomatidine can actually prevent the emergence of bacteria with decreased susceptibility to aminoglycosides, we followed the regrowth of bacteria in cultures exposed to various concentrations of gentamicin alone or in combination with tomatidine at 8 mg/L. Figure 2(d) further demonstrates that the presence of tomatidine

could significantly decrease the number of cfu recovered from cultures exposed to concentrations of gentamicin ranging from 0.5 to 2 mg/L for 24 h (unpaired *t*-test between control and tomatidine-exposed conditions for each gentamicin concentration). From these time-kill experiments, isolated colonies obtained from cultures exposed to gentamicin combined or not with 8 mg/L of tomatidine were analysed for their susceptibility to gentamicin. When ATCC 29213 was exposed to gentamicin alone, the emergence of numerous normal-growing isolates showing decreased susceptibility to gentamicin (MIC ranging from 1 to 4 mg/L) was easily detected. Such an exposure to gentamicin also yielded numerous isolates with the SCV phenotype showing the expected reduced susceptibility to gentamicin (MIC ranging from 4 to 8 mg/L). It is noteworthy that these SCVs maintained their high susceptibility to tomatidine (MIC <0.5 mg/L). Besides, although much fewer colonies were recovered from cultures exposed to the various combinations of gentamicin and tomatidine (Figure 2d), normal-growing isolates with decreased susceptibility to gentamicin were found as seen with the gentamicin alone in cultures. Interestingly, small pinpoint colonies recovered from the combination cultures were particularly unstable and easily reverted to the normal-growing phenotype when sub-cultured. It was therefore not possible to adequately measure the susceptibility of these SCVs to gentamicin or tomatidine and see if resistance of SCVs to tomatidine could develop from exposure to the combination. Finally, it is important to mention that the normal-growing isolates having reduced susceptibility to gentamicin (recovered from either the gentamicin or the gentamicin-tomatidine combination cultures) were still subject to the potentiating effect of tomatidine although it required about two times more gentamicin to attain the level of growth inhibition observed with the parental strain ATCC 29213, *i.e.*, a concentration of gentamicin related to the new MIC.

Overall, the results in this section clearly demonstrate that tomatidine potentiates the bactericidal action of aminoglycoside antibiotics against *S. aureus* and suggest that tomatidine can also reduce the emergence of bacteria with decreased susceptibility to aminoglycosides.

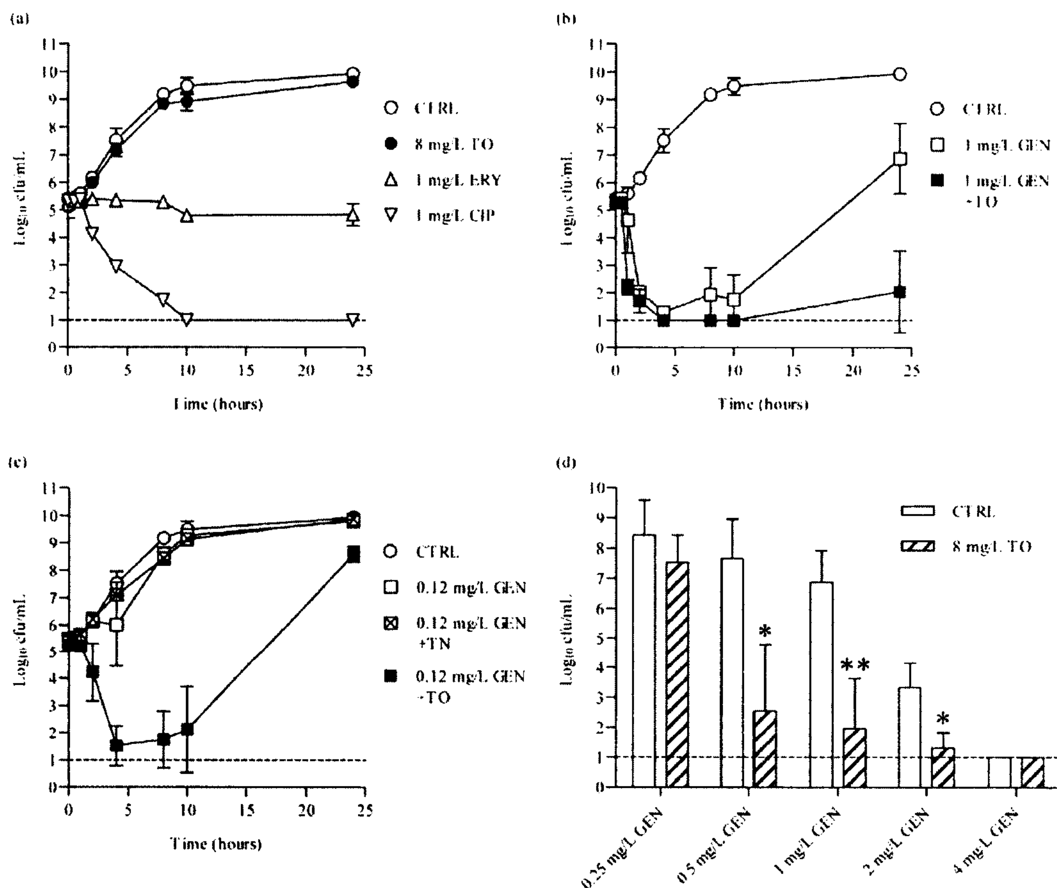

FIGURE 2. Tomatidine (TO) potentiates the bactericidal activity of aminoglycoside antibiotics against ATCC 29213. (a) Time-kill experiments showing the viability of ATCC 29213 untreated or in the presence of 8 mg/L of TO, 1 mg/L of erythromycin (ERY) and 1mg/L of ciprofloxacin (CIP). (b) Time-kill experiments showing the viability of ATCC 29213 untreated or in the presence of 1 mg/L of gentamicin (GEN) in the absence or in the presence of 8 mg/L of TO. (c) Time kill-experiments showing the viability of ATCC 29213 untreated or in the presence of 0.12 mg/L of gentamicin (GEN) in the absence or presence of 8 mg/L of TO or tomatine (TN). The cfu detection limit is indicated by a dotted line and corresponds to 10 cfu/mL. (d) cfu/mL recovered from 24-h cultures treated with GEN at concentrations ranging from 0.25 to 4 mg/L combined or not with 8 mg/L of TO. Significant differences between the CTRL and TO conditions are shown (**, $P < 0.01$; *, $P < 0.05$;

unpaired *t*-test). Data are presented as means with standard deviations from at least two independent experiments.

Tomatidine affects the hemolytic ability of *S. aureus* and represses several *agr*-regulated virulence factors. The quorum-sensing dependent *agr* system is known to repress the expression of many cell-surface proteins while allowing expression of several exoproteins.[17, 18] Importantly, the ability of *S. aureus* to lyse red blood cells is regulated by the *agr* system.[17] This association between hemolysis and the activity of *agr* was used to easily detect if tomatidine could have an impact on the virulence of *S. aureus*. Figure 3(a) shows that inclusion of tomatidine in blood agar plates strikingly reduced the hemolytic activity of all *S. aureus* strains tested, whereas tomatine did not. It is noteworthy that NewbouldΔ*sigB* and 8325-4 are strains with dysfunctional SigB activity, resulting in increased *agr* activity and, consequently, in an increase in hemolytic ability.[27, 41-43] Figure 3(a) shows that both the increased hemolytic activity of these strains and the hemolytic activity of their counterpart strains having a functional *sigB* operon (Newbould and SH1000, respectively) were inhibited by tomatidine.

Quantitative PCR was then used to measure the expression of *hld* (encoding the δ-toxin and a fragment of RNAIII, the effector of the *agr* system)[18] in ATCC 29213 treated with different concentrations of tomatidine. As expected, the expression of *hld* was decreased as a function of tomatidine concentration (Figure 3b). Figure 3(c) shows the effects of tomatidine and tomatine at 12.8 mg/L on the expression of some of the *agr*-upregulated genes (*hla*, *hld*, *geh*, *nuc*, *plc* and *splC*).[17] As expected, tomatidine significantly repressed the expression of the *agr*-upregulated genes whereas tomatine did not (one-tailed unpaired *t*-test between tomatidine- and tomatine-exposed conditions for each gene). These results demonstrate that exposure to tomatidine represses several virulence factors in *S. aureus*.

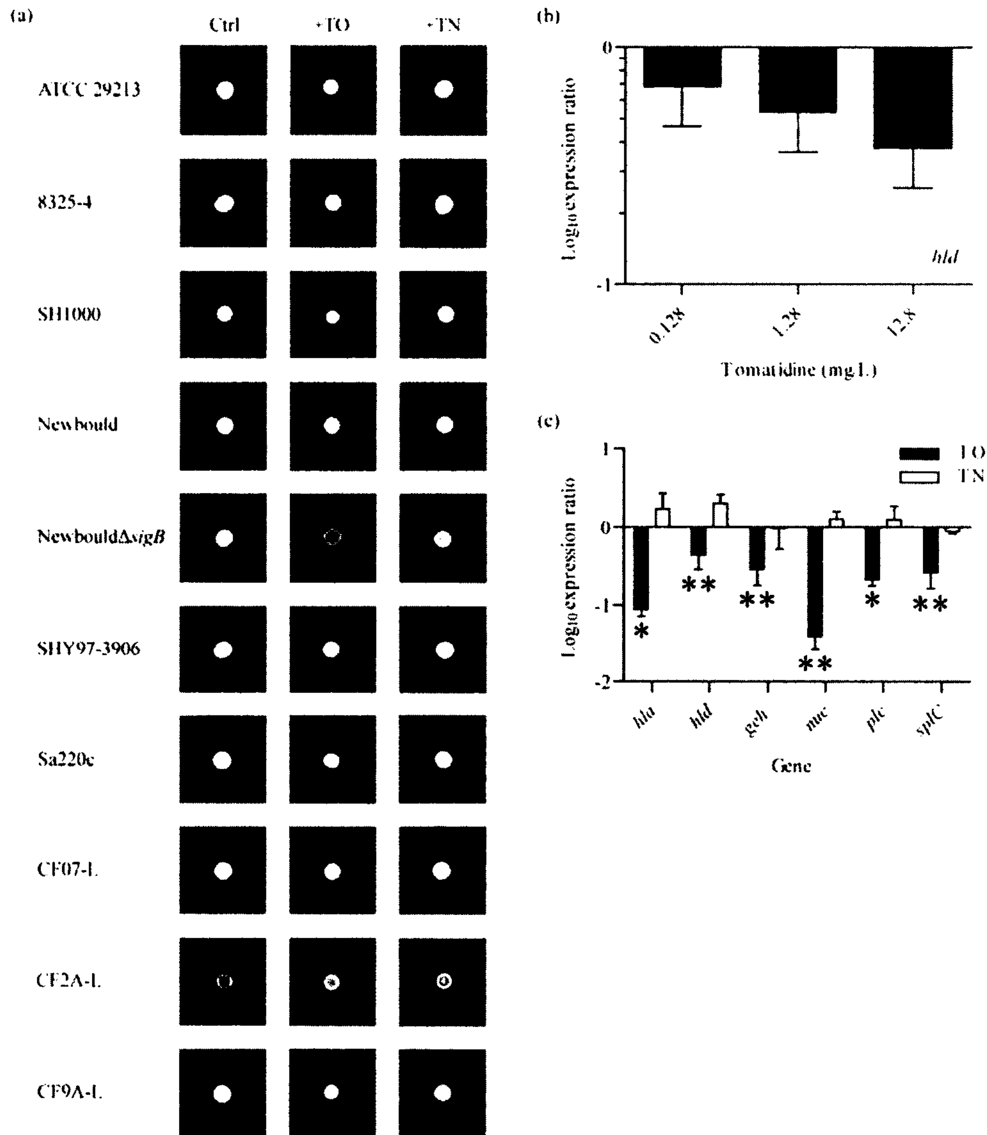

FIGURE 3. Tomatidine (TO) alters the expression of *agr*-regulated virulence factors in *S. aureus*. (a) Haemolytic ability of several strains in the absence or presence of 8 mg/L of TO or tomatine (TN). (b) Concentration-dependent effect of TO on the expression of *hld* (encoding the δ-hemolysin and a fragment of RNAIII, the effector of the *agr* system). Data are presented as means with standard deviations from at least three independent experiments. (c) Effect of 12.8 mg/L of TO or TN on the expression of the *agr*-regulated genes *hla*, *hld*, *geh*, *nuc*, *plc*

and *splC*. Significant differences between the TO and TN conditions for the expression of each gene are shown (**, $P < 0.01$; *, $P < 0.05$; one-tailed unpaired *t*-test). Data are presented as means with standard deviations from at least two independent experiments. Ctrl, control.

DISCUSSION

This study demonstrates that the plant product tomatidine specifically potentiates the bactericidal activity of aminoglycoside antibiotics against *S. aureus* and also more broadly against staphylococci. This effect was documented for several strains of diverse clinical origins and included multi-resistant *S. aureus* strains. Surprisingly, tomatidine also potentiates the activity of aminoglycoside antibiotics against aminoglycoside-resistant bacteria carrying AMEs although the MICs against such strains did not drop below the resistance breakpoints. Although we could not demonstrate a potentiating effect of tomatidine on the inhibitory activity of aminoglycosides against *P. aeruginosa*, *E. coli* or even *Enterococcus spp.*, it remains to be seen whether such a biological activity can be observed in other clinically relevant bacterial genera.

A major challenge in the clinical use of aminoglycosides is to empirically evaluate the dose and administration schedule needed to obtain a non-toxic, but still successful, therapy.[44] If the development of tomatidine as an adjunct therapy is successful eventually, the use of tomatidine in combination with aminoglycoside antibiotics could allow new efficient treatment regimens with reduced aminoglycoside amounts and a lower risk of drug toxicity. Moreover, the emergence of bacteria that are less susceptible after exposure to aminoglycosides has been fully documented by others[39, 40] and the results from this study also suggest that the presence of tomatidine could reduce the incidence of bacteria that are less

susceptible to aminoglycosides. Thus, an aminoglycoside potentiator such as tomatidine could possibly extend the clinical applications and safe usage of aminoglycosides.

In addition to the potentiating effect of tomatidine demonstrated here on the inhibitory activity of aminoglycosides against prototypical *S. aureus*, we recently reported that tomatidine also has a specific bacteriostatic activity against *S. aureus* SCVs.[20] More importantly, we showed that tomatidine has the ability to inhibit the replication of SCVs internalized in CF-like human airway epithelial cells.[20] Because *S. aureus* of the normal and SCV phenotypes are often recovered simultaneously from CF patients[22, 23] and because aminoglycoside antibiotics are already used in the management of bacterial infections in CF,[10, 45, 46] this suggests that a combination therapy of tomatidine and aminoglycosides could be especially effective in the context where *S. aureus* and *S. aureus* SCVs co-infect a CF patient. Aminoglycoside antibiotics have a wide spectrum of antimicrobial activity that affects both Gram-positive and Gram-negative pathogens,[47] and in this new era plagued by multi-resistant organisms[48] the revitalization of a well-known class of antibiotics by potentiators such as tomatidine would certainly have its merit.

The mechanism by which tomatidine increases the activity of aminoglycoside antibiotics against prototypical *S. aureus* is not yet understood. It is possible that tomatidine increases the uptake of aminoglycoside-antibiotics by increasing the permeability of *S. aureus*. This would explain the improved bactericidal activity of the combination of tomatidine with subinhibitory concentrations of aminoglycosides since the activity of such antibiotics is intimately associated with their uptake into bacteria.[38] Inversely, it is also possible that subinhibitory concentrations of aminoglycosides allow tomatidine to reveal its own antimicrobial activity against *S. aureus*. As an example, it is known that the action of aminoglycosides causes channel formation in bacterial membranes that support influx of antibiotics,[38] and this may allow the uptake of tomatidine to occur. Tomatidine could then act on protein synthesis as

previously anticipated.[20] We currently tend to rule out that the action of tomatidine on *S. aureus* is through membrane permeabilization because of the lack of activity of the structurally related tomatine, the lack of potentiating activity of tomatidine on other antibiotic classes against *S. aureus* and the inability of tomatidine to potentiate aminoglycoside activity against both hyperpermeable, LPS-rough and efflux-pump-deficient *E. coli* strains.

The inhibition of *S. aureus* hemolytic activity we noticed in the presence of tomatidine was associated with the inability of the bacterium to activate the quorum-sensing *agr* system, which normally up-regulates the expression of several virulence factors, such as α-hemolysin and other exoproteins.[17, 18] Accordingly, we demonstrated that tomatidine inhibits the expression of several genes known to be up-regulated by the *agr* system. Our results thus suggest that tomatidine could alter the virulence of *S. aureus* by repressing the expression of several *agr*-upregulated genes. Here again, the mechanism by which tomatidine acts on the *agr* system is not known, but it is possible that tomatidine influences the production of virulence factors by interacting with the bacterial surface and/or interfering with signal transduction, as proposed for other virulence inhibitors in staphylococci.[49-52]

Overall, this study demonstrates that tomatidine exerts different biological effects on *S. aureus* that are likely to be useful once translated into therapeutic applications. We have shown here that the synergy between tomatidine and aminoglycosides could help reduce the amounts of these drugs required for a bactericidal effect against prototypical *S. aureus* while also reducing the incidence of resistance. This synergy, in addition to the standalone inhibitory activity of tomatidine previously reported against extracellular and intracellular *S. aureus* SCVs, remains to be confirmed *in vivo*, however. In the meantime, tomatidine now represents an interesting new structural scaffold on which to base future investigations aiming at the development of anti-staphylococcal agents efficient against strains causing chronic and difficult-to-treat infections.

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TRANSPARENCY DECLARATIONS

None to declare.

REFERENCES

1. Shah PM. The need for new therapeutic agents: what is the pipeline? *Clin Microbiol Infect* 2005; **11 Suppl 3**: 36-42.
2. Talbot GH, Bradley J, Edwards JE, Jr. *et al.* Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clin Infect Dis* 2006; **42**: 657-668.
3. Witte W, Cuny C, Klare I *et al.* Emergence and spread of antibiotic-resistant Gram-positive bacterial pathogens. *Int J Med Microbiol* 2008; **298**: 365-377.
4. Archer GL. *Staphylococcus aureus*: a well-armed pathogen. *Clin Infect Dis* 1998; **26**: 1179-1181.
5. Casey AL, Lambert PA, Elliott TS. Staphylococci. *Int J Antimicrob Agents* 2007; **29 Suppl 3**: S23-32.
6. Goerke C, Wolz C. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. *Int J Med Microbiol* 2004; **294**: 195-202.
7. Canadian Cystic Fibrosis Foundation Patient Data Registry Report 2009. http://www.cysticfibrosis.ca/assets/files/pdf/CPDR_ReportE.pdf (27 October 2011, date last accessed).
8. Cystic Fibrosis Foundation Patient Registry Annual Data Report 2009. http://www.cff.org/UploadedFiles/research/ClinicalResearch/Patient-Registry-Report-2009.pdf (27 October 2011, date last accessed).
9. European Cystic Fibrosis Society Patient Registry Report 2007 Data. http://www.ecfs.eu/files/webfm/webfiles/File/ecfs_registry/ECFRreport2007final.pdf (27 October 2011, date last accessed).
10. Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 2002; **15**: 194-222.

11. Parkins MD, Elborn JS. Newer antibacterial agents and their potential role in cystic fibrosis pulmonary exacerbation management. *J Antimicrob Chemother* 2010; **65**: 1853-1861.

12. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol* 2009; **7**: 629-641.

13. Dasenbrook EC, Checkley W, Merlo CA *et al.* Association between respiratory tract methicillin-resistant *Staphylococcus aureus* and survival in cystic fibrosis. *Jama* 2010; **303**: 2386-2392.

14. Dasenbrook EC, Merlo CA, Diener-West M *et al.* Persistent methicillin-resistant *Staphylococcus aureus* and rate of FEV1 decline in cystic fibrosis. *Am J Respir Crit Care Med* 2008; **178**: 814-821.

15. Leonard FC, Markey BK. Meticillin-resistant *Staphylococcus aureus* in animals: a review. *Vet J* 2008; **175**: 27-36.

16. García-Álvarez L, Holden MT, Lindsay H *et al.* Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis* 2011; **11**: 595-603.

17. Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* 2003; **48**: 1429-1449.

18. Novick RP, Geisinger E. Quorum sensing in staphylococci. *Annu Rev Genet* 2008; **42**: 541-564.

19. González-Lamothe R, Mitchell G, Gattuso M *et al.* Plant antimicrobial agents and their effects on plant and human pathogens. *Int J Mol Sci* 2009; **10**: 3400-3419.

20. Mitchell G, Gattuso M, Grondin G *et al.* Tomatidine inhibits replication of *Staphylococcus aureus* small-colony variants in cystic fibrosis airway epithelial cells. *Antimicrob Agents Chemother* 2011; **55**: 1937-1945.

21. Niño J, Correa YM, Mosquera OM. Biological activities of steroidal alkaloids isolated from *Solanum leucocarpum*. *Pharm. Biol.* 2009; **47**: 255-259.

22. Proctor RA, von Eiff C, Kahl BC *et al.* Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* 2006; **4**: 295-305.
23. Kahl BC, Duebbers A, Lubritz G *et al.* Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. *J Clin Microbiol* 2003; **41**: 4424-4427.
24. Tuchscherr L, Medina E, Hussain M *et al.* *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO Mol Med* 2011; **3**: 129-141.
25. Diarra MS, Petitclerc D, Lacasse P. Response of *Staphylococcus aureus* isolates from bovine mastitis to exogenous iron sources. *J Dairy Sci* 2002; **85**: 2141-2148.
26. Moisan H, Brouillette E, Jacob CL *et al.* Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *J Bacteriol* 2006; **188**: 64-76.
27. Horsburgh MJ, Aish JL, White IJ *et al.* σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* 2002; **184**: 5457-5467.
28. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-First Informational Supplement M100-S21. CLSI, Wayne, PA, USA, 2011.
29. Eliopoulos GM, Moellering RC. Antimicrobial combinations. In: Lorian V, ed. *Antibiotics in Laboratory Medicine*. Baltimore, MD: Williams and Wilkins, 1996; 330-336.
30. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother* 2003; **52**: 1.
31. Schmitz FJ, Fluit AC, Gondolf M *et al.* The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *J Antimicrob Chemother* 1999; **43**: 253-259.

32. Allard M, Moisan H, Brouillette E *et al.* Transcriptional modulation of some *Staphylococcus aureus* iron-regulated genes during growth *in vitro* and in a tissue cage model *in vivo*. *Microbes Infect* 2006; **8**: 1679-1690.

33. Sampson BA, Misra R, Benson SA. Identification and characterization of a new gene of *Escherichia coli* K-12 involved in outer membrane permeability. *Genetics* 1989; **122**: 491-501.

34. Mazzariol A, Cornaglia G, Nikaido H. Contributions of the AmpC β-lactamase and the AcrAB multidrug efflux system in intrinsic resistance of *Escherichia coli* K-12 to β-lactams. *Antimicrob Agents Chemother* 2000; **44**: 1387-1390.

35. Pazoles CJ, Kulpa CF, Jr. Biosynthesis and structure of lipopolysaccharide in an outer membrane-defective mutant of *Escherichia coli* J5. *Biochim Biophys Acta* 1977; **466**: 160-175.

36. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resist Updat* 2010; **13**: 151-171.

37. Shaw KJ, Rather PN, Hare RS *et al.* Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* 1993; **57**: 138-163.

38. Davis BD. Mechanism of bactericidal action of aminoglycosides. *Microbiol Rev* 1987; **51**: 341-350.

39. Miller MH, Wexler MA, Steigbigel NH. Single and combination antibiotic therapy of *Staphylococcus aureus* experimental endocarditis: emergence of gentamicin-resistant mutants. *Antimicrob Agents Chemother* 1978; **14**: 336-343.

40. Wilson SG, Sanders CC. Selection and characterization of strains of *Staphylococcus aureus* displaying unusual resistance to aminoglycosides. *Antimicrob Agents Chemother* 1976; **10**: 519-525.

41. Cheung AL, Chien YT, Bayer AS. Hyperproduction of α-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*. *Infect Immun* 1999; **67**: 1331-1337.

42. Herbert S, Ziebandt AK, Ohlsen K *et al.* Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect Immun* 2010; **78**: 2877-2889.

43. Lauderdale KJ, Boles BR, Cheung AL *et al.* Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. *Infect Immun* 2009; **77**: 1623-1635.

44. Drusano GL, Ambrose PG, Bhavnani SM *et al.* Back to the future: using aminoglycosides again and how to dose them optimally. *Clin Infect Dis* 2007; **45**: 753-760.

45. Høiby N. Recent advances in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. *BMC Med* 2011; **9**: 32.

46. Prayle A, Smyth AR. Aminoglycoside use in cystic fibrosis: therapeutic strategies and toxicity. *Curr Opin Pulm Med* 2010; **16**: 604-610.

47. Durante-Mangoni E, Grammatikos A, Utili R *et al.* Do we still need the aminoglycosides? *Int J Antimicrob Agents* 2009; **33**: 201-205.

48. Wise R. The urgent need for new antibacterial agents. *J Antimicrob Chemother* 2011; **66**: 1939-1940.

49. Balaban N, Cirioni O, Giacometti A *et al.* Treatment of *Staphylococcus aureus* biofilm infection by the quorum-sensing inhibitor RIP. *Antimicrob Agents Chemother* 2007; **51**: 2226-2229.

50. Gov Y, Bitler A, Dell'Acqua G *et al.* RNAIII inhibiting peptide (RIP), a global inhibitor of *Staphylococcus aureus* pathogenesis: structure and function analysis. *Peptides* 2001; **22**: 1609-1620.

51. Lyon GJ, Mayville P, Muir TW *et al.* Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. *Proc Natl Acad Sci U S A* 2000; **97**: 13330-13335.

52. Projan SJ, Brown-Skrobot S, Schlievert PM *et al.* Glycerol monolaurate inhibits the production of β-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. *J Bacteriol* 1994; **176**: 4204-4209.

DISCUSSION

Les nombreux efforts de recherche entourant les SCVs de *S. aureus* sont justifiés par leur association avec les infections chroniques (Proctor *et al.*, 2006). Cette association est particulièrement observée chez les patients FK affectés par des infections pulmonaires chroniques à *S. aureus* (Kahl *et al.*, 1998; Kahl *et al.*, 2003b). Une étude clinique réalisée dernièrement au laboratoire du Pr François Malouin montre que les SCVs sont isolés d'environ 23% des patients FK traités à la Clinique de fibrose kystique du Centre hospitalier universitaire de Sherbrooke et n'a révélé aucun effet détectable des SCVs sur la fonction pulmonaire des patients malgré la plus grande habileté de ces souches à former du biofilm (voir Annexe 2, Résumé 1). Cependant, cette étude n'offrait que des images instantanées de la situation des différents patients et une étude longitudinale évaluant l'effet à long terme de la colonisation des poumons FK par les SCVs est nécessaire avant de porter une conclusion. Il est aussi possible que les SCVs contribuent à la progression de la maladie sans toutefois influencer directement la fonction pulmonaire. Par exemple, le biofilm des SCVs pourrait promouvoir ou interférer avec les infections à *P. aeruginosa* ou bien constituer un réservoir de bactéries récalcitrantes aux antibiothérapies. Bien que plusieurs des propriétés *in vitro* des SCVs (*p.ex.* leur moins grande susceptibilité à certains antibiotiques, leur persistance à l'intérieur des cellules non phagocytaires et leur habileté à former du biofilm) supportent l'hypothèse d'une virulence dirigée vers l'établissement d'infections persistantes, il n'a jamais été clairement démontré que les infections aux SCVs ont bel et bien une tendance significative à la chronicité. Il est toutefois envisageable que le phénotype SCV soit optimisé à la coexistence avec son hôte en évitant la destruction tissulaire et l'activation du système immunitaire (Kipp *et al.*, 2005; Proctor *et al.*, 1995; Proctor *et al.*, 2006; Tuchscherr *et al.*, 2010; Tuchscherr *et al.*, 2011), expliquant l'absence d'impact des SCVs sur la fonction pulmonaire des patients FK.

Une des découvertes récentes dans le domaine des SCVs et de la FK fut la démonstration que la présence de *P. aeruginosa* favorise l'apparition de SCVs chez *S. aureus* (Hoffman *et al.*, 2006) et, par conséquent, la formation de biofilm (Chapitre 3). Ces dernières études ont été effectuées avec des souches types de *P. aeruginosa* et seulement quelques souches de *S. aureus*. Une des études actuellement en cours dans le laboratoire du Pr François Malouin a pour but de caractériser la diversité des interactions interspécifiques entre plusieurs isolats (et co-isolats) FK de ces deux pathogènes ainsi que de déterminer les mécanismes moléculaires impliqués dans leur coexistence. Une des conclusions émergeant de ces études est que les isolats FK de *S. aureus* montrent une très grande diversité de réponses aux isolats de *P. aeruginosa*, ce qui suggère que la complexité des interactions entre ces deux pathogènes est plus grande que ce qui était initialement suspecté (voir Annexe 2, Résumé 2). De plus, aucune association entre l'émergence du phénotype SCV et la co-infection avec *P. aeruginosa* ne se dégage des études cliniques menées actuellement au laboratoire du Pr François Malouin (voir Annexe 2, Résumé 1). L'hypothèse actuelle est que le *S. aureus* persistant dans les voies respiratoires CF acquiert des mutations conférant une résistance aux exoproduits de *P. aeruginosa* lors d'une co-infection. Bien que nous ne possédions pas encore d'évidence que de telles mutations sont présentes chez les isolats FK de *S. aureus*, il est concevable que des mutations dans la chaîne de transport des électrons puissent empêcher les exoproduits de *P. aeruginosa* de lier leur cible (Voggu *et al.*, 2006). Il est aussi possible que des mutations dans certains facteurs ou régulateurs de virulence empêchent *S. aureus* de former des SCVs et du biofilm en réponse à ces exoproduits. Il sera aussi important de montrer que la présence de souches de *P. aeruginosa* peut bel et bien mener à la formation de SCVs et à la modulation de la virulence de *S. aureus* dans des modèles d'infections expérimentales. Ainsi, le nombre de CFU au phénotype normal et SCV pourrait être déterminé chez *S. aureus* lors de co-infections avec *P. aeruginosa* (Voir méthodologie Chapitre 2). De plus, l'expression de certains gènes pourrait être suivie (*p. ex.* à l'aide du qPCR) afin d'établir plus précisément l'effet de *P. aeruginosa* sur la virulence de *S. aureus in vivo*. L'étude de la pathogenèse de *S. aureus* se doit d'être effectuée en considérant le contexte souvent polymicrobien des voies respiratoires FK (voir Annexe 1).

Plusieurs autres facteurs favorisent l'émergence du phénotype SCV tels que l'exposition à certaines classes d'antimicrobiens (Proctor *et al.*, 2006), notamment les antibiotiques de la classe des aminoglycosides, ce qui a été confirmé *in vitro* et *in vivo* dans le chapitre 2. L'utilisation d'antibiotiques, et plus particulièrement d'aminoglycosides, est courante dans le traitement des infections bactériennes chez les patients FK (Lyczak *et al.*, 2002). Cependant, aucune association entre l'émergence du phénotype SCV et les traitements antibiotiques n'est encore dégagée des études cliniques menées au laboratoire du Pr Malouin (voir Annexe 2, Résumé 1). Comme mentionné précédemment, l'étude menée ne représente que des images instantanées de la situation des patients et une étude longitudinale est requise afin de savoir si certains antibiotiques peuvent favoriser l'émergence du phénotype SCV. Il est toujours possible qu'un autre facteur retrouvé dans les poumons des patients FK favorise l'émergence de SCVs. Par exemple, dans le contexte hypothétique où certains peptides antimicrobiens cationiques seraient moins actifs dans les voies respiratoires FK (Gibson *et al.*, 2003; Smith *et al.*, 1996), il est possible que des doses sous-inhibitrices de ces peptides soient alors présentes dans l'environnement de la bactérie et favorisent l'émergence de SCVs (Sadowska *et al.*, 2002). Il est aussi possible que le phénotype SCV soit tout simplement une adaptation à l'environnement anaérobique rencontré dans les voies respiratoires FK puisque les SCVs n'utilisent pas leur chaîne de transport des électrons. Finalement, les métabolites requis au fonctionnement de la chaîne de transport des électrons sont peut-être en abondance dans les voies respiratoires FK, assurant aux SCVs un métabolisme normal au cours de l'infection.

Comme mentionné dans l'introduction, relativement peu des connaissances actuelles sur les SCVs proviennent d'isolats cliniques à cause de leur grande instabilité phénotypique. Bien qu'il soit souvent considéré que le phénotype SCV pourrait être strictement causé par des mutations dans des gènes impliqués dans la chaîne de transport des électrons ou dans la biosynthèse de la thymidine, peu d'études décrivent précisément de telles mutations dans des souches cliniques (Chatterjee *et al.*, 2008; Lannergard *et al.*, 2008). Les récentes avancées technologiques permettant le séquençage d'ADN à haut débit vont permettre de comparer le

génome de plusieurs paires d'isolats cliniques génétiquement liés au phénotype normal et SCV afin de trouver les potentielles mutations expliquant le phénotype SCV. Cependant, dans le cas des SCVs au phénotype très instable, il faudra trouver le moyen de séquencer le génome bactérien à partir de petites quantités de matériel biologique afin de s'assurer d'avoir des résultats représentatifs de l'isolat tel qu'il était chez le patient.

La fréquence avec laquelle certains isolats cliniques passent d'un phénotype SCV à un phénotype normal (et vice versa) peut suggérer que des mécanismes de régulation ou génétiques sont impliqués dans l'acquisition et la perte de ce phénotype (Massey *et al.*, 2001). L'implication du facteur SigB dans l'acquisition du phénotype SCV est la première démonstration que l'émergence du phénotype SCV est un phénomène régulé chez *S. aureus* (Chapitre 2 et 3), ce qui est d'autant plus intéressant en considérant que SigB est un facteur de transcription activable par différents stress. L'idée que le phénotype SCV est spécialisé dans la réponse à certains stress est donc envisageable. Le mécanisme complet de formation des SCVs dépendant de l'activation de SigB reste cependant à élucider. Il est possible que SigB soit impliqué dans la régulation de la transposition de séquences d'insertion à l'intérieur de gènes liés à la chaîne de transport des électrons comme il a été démontré pour des gènes impliqués dans la formation de biofilm (Valle *et al.*, 2007). Il est aussi possible que l'activité du système antitoxine/toxine MazEF (dont l'expression est influencée par SigB) puisse conduire à la formation de SCVs en réponse à différents stress (Fu *et al.*, 2007; Gertz *et al.*, 1999; Senn *et al.*, 2005b). Une interaction avec des systèmes influençant la mutabilité bactérienne (Schaaff *et al.*, 2003) ou l'acquisition de modifications épigénétiques contrôlant l'expression de gènes demeurent des avenues à explorer. Une étude récente a démontrée que l'inversion réversible d'une grande partie de génome (*flip-flop inversion*) permet à *S. aureus* de passer rapidement d'un phénotype normal à un phénotype SCV (et vice-versa) et que cette inversion est régulée par RecA (Cui *et al.*, 2012). Dans le cas où la seule activité de SigB ou d'autres régulateurs puissent conférer le phénotype SCV à la bactérie, le séquençage de génomes ne pourra pas répondre à nos questions. Il serait alors pertinent de moduler artificiellement l'activité de SigB

en surexprimant des gènes impliqués dans la régulation de ce facteur, notamment RsbU (Pane-Farre *et al.*, 2009). Il serait également intéressant de mener une étude utilisant des techniques de génération de collections de mutants afin d'obtenir une vue plus globale des gènes potentiellement impliqués dans l'acquisition du phénotype SCV.

L'activité de SigB n'est pas seulement impliquée dans l'acquisition du phénotype SCV puisqu'elle a un rôle central et constitutif dans la régulation de l'expression de plusieurs gènes chez ces variants (Chapitre 1). Cependant, dans la littérature, un rôle plus important semble habituellement attribué à l'absence d'activation du système *agr* dans le contrôle de l'expression des facteurs de virulence chez les SCVs (Proctor *et al.*, 2006), l'hypothèse étant que la faible croissance des SCVs ne permet pas l'atteinte de la concentration critique de peptides auto-inducteurs requise à l'autoactivation de ce système. Mes résultats ont bel et bien démontré que la complémentation de la croissance d'un SCV auxotrophe pour la ménadione active le système *agr* et réprime l'activité de SigB (Chapitre 2). Cependant, des résultats plus récents supportent l'idée que SigB pourrait être le facteur dominant dans le contrôle de la virulence des SCVs (voir Annexe 3). Tandis que l'inactivation génétique de SigB influence fortement la formation de biofilm et l'activité nucléase des SCVs, la surexpression de RNAIII dans un SCV affecte peu ou pas ces phénotypes (voir Annexe 3, Figure 2, 3 et 6). De plus, le niveau d'expression de RNAIII semble fortement contrôlé par l'activité SigB (voir Annexe 3, Figure 3). Il serait cependant pertinent d'effectuer des expériences contrôles consistant à évaluer l'effet de la surexpression d'*agrA* et d'*agrACDB* chez CF07-S afin de confirmer que l'activation du système *agr* n'a pas (ou peu) d'influence sur la formation de biofilm et l'activité nucléase des SCVs par une voie indépendante de RNAIII (Queck *et al.*, 2008). Il aurait aussi été pertinent de faire un double mutant CF07-SΔ*sigB*Δ*agr* afin d'étudier plus en détail l'interaction entre SigB et le système *agr* dans le contrôle de la virulence des SCVs. Bien que toujours sujet à la controverse, une association entre le niveau énergétique de la bactérie et l'activité SigB reste toujours possible (Proctor et von Humboldt, 1998) et pourrait

expliquer pourquoi et comment SigB se retrouve en amont du contrôle de l'expression des facteurs de virulence chez les SCVs.

En lien avec l'activité des régulateurs globaux, plusieurs protéines de surface sont activement exprimées chez les SCVs comparativement aux souches normales (*p. ex.* FnBPA, ClfA, SceD) tandis que plusieurs facteurs sécrétés sont réprimés (Moisan *et al.*, 2006). Une des hypothèses supportées par mes travaux de doctorat est que les SCVs isolés des patients FK sont verrouillés dans un état de colonisation par une expression constitutive d'adhésines. Il a été montré que l'expression de *fnbA* est soutenue à toutes les phases de croissance chez les SCVs et la spectroscopie de force atomique a été utilisée afin de confirmer que les SCVs lient effectivement la fibronectine d'une façon accrue et soutenue, et cela d'une façon dépendante de l'activité de SigB (Chapitre 1). En plus de la régulation de l'expression des protéines de surface, il est connu que les protéases sécrétées par *S. aureus* sont utilisées pour dégrader les adhésines et ainsi réguler l'adhésion bactérienne à des ligands spécifiques (Karlsson *et al.*, 2001). L'activité protéolytique presque nulle des SCVs isolées de patients FK (voir Annexe 3, Figure 1) supporte aussi cette hypothèse de spécialisation phénotypique des SCVs favorisant la colonisation des tissus de l'hôte.

L'étude utilisant la spectroscopie de force atomique a aussi permis de supporter la fonction de l'interaction FnBPs-fibronectine dans la formation d'une adhésion aux tissus de l'hôte considérablement résistante aux tensions mécaniques, ce qui est en accord avec des études montrant que les FnBPs sont importants à la colonisation tissulaire sous des forces de cisaillement (Brouillette *et al.*, 2003b; Reddy et Ross, 2001). Cette étude ne permet évidemment pas de conclure de l'importance relative de l'interaction des protéines de surfaces de *S. aureus* avec d'autres ligands que la fibronectine. Cependant, elle permet de conclure que les FnBPs sont les déterminants majeurs de la formation d'une interaction résistante aux tensions mécaniques avec la fibronectine. Étant donné qu'une seule population de force

spécifique à l'interaction fibronectine-*S. aureus* a été observée et que l'inactivation génétique de *fnbAB* a presqu'abolie la présence de cette dernière, il semble que les autres adhésines liants la fibronectine sont soit retrouvées à faible fréquence chez les souches à l'étude ou sont impliquées dans des interactions dissimulées par le bruit de fond de la technique.

Bien que la capacité accrue à former du biofilm ait déjà été soulignée chez des SCVs d'autres espèces bactériennes (Al Laham *et al.*, 2007; Haussler, 2004; von Gotz *et al.*, 2004), la recherche a surtout été dirigée vers l'habileté des SCVs à infecter les cellules non phagocytaires chez *S. aureus* (Proctor *et al.*, 2006; Sendi et Proctor, 2009). Il semble cependant que les SCVs de *S. aureus* soient bel et bien d'excellents producteurs de biofilm (Chapitre 2 et 3) et le rôle potentiel que ce facteur joue dans l'établissement d'infections chroniques ne doit pas être négligé, surtout dans le contexte d'infections pulmonaires chez les patients FK (Costerton *et al.*, 1999; Davies et Bilton, 2009; Hoiby *et al.*, 2010; Stewart, 2002). Jusqu'à maintenant, les mécanismes impliqués dans la formation de biofilm des SCVs étaient méconnus, bien que certains résultats semblent suggérer une implication de PIA (Al Laham *et al.*, 2007; Singh *et al.*, 2010a). Cependant, le rôle que joue SigB, *agr* et les FnBPs dans la formation de biofilm des souches normales suggèrent que les SCVs devraient former un biofilm dépendant des protéines de surface. Mes résultats montrent très clairement que le biofilm des SCVs est constitué de protéines (voir Annexe 3, Figure 1) et que l'interaction entre SigB et le système *agr* contrôle la formation d'un biofilm en permettant une expression accrue et constante de FnBPA (voir Annexe 3, Figures 2, 3 et 4). Mes résultats suggèrent aussi que l'ADN extracellulaire est une autre composante importante du biofilm des SCVs, car l'activité autolytique des SCVs est accrue et que SigB inhibe leur activité nucléase (voir Annexe 3, Figures 5 et 6). Il est intéressant de constater que les MRSA utilisent un mécanisme similaire de formation de biofilm et que la formation d'un biofilm dépendant de protéines extracellulaires est habituellement accompagnée d'une absence de production de PIA chez ces souches (O'Neill *et al.*, 2008; O'Neill *et al.*, 2007; Pozzi *et al.*, 2012). Il reste à savoir si d'autres protéines de surface et régulateurs de virulence sont impliqués dans la formation du

biofilm chez les SCVs. Il serait aussi pertinent d'étudier la formation de biofilm des SCVs en contexte polymicrobien (*p. ex.* en présence de *P. aeruginosa*) et en utilisant des techniques plus raffinées (*p. ex.* les chambres à flot et la microscopie confocale).

Certaines études ont démontré que l'internalisation de *S. aureus* dans les cellules non phagocytaires est influencée par SigB (Moisan *et al.*, 2006; Nair *et al.*, 2003) et les FnBPs (Sinha et Herrmann, 2005). Cependant, aucune différence d'internalisation significative n'a été observée entre une souche normale et un SCV dans les cellules Calu-3 (Chapitre 4). De plus, bien qu'une différence marquée d'internalisation ait été observée entre 8325-4 et DU5883 (Δ*fnbAB*), aucune différence d'internalisation significative n'a été observée entre CF07-S, CF07-SΔ*sigB* et CF07-SΔ*fnbA* dans les cellules FK dérivées des Calu-3 (voir Annexe 3, Figure 7). L'hypothèse la plus simple est que la seule expression de FnBPB assure déjà un certain niveau d'internalisation aux différentes souches et que l'expression accrue de FnBPA chez les SCVs isolés des patients FK a plutôt pour fonction de conférer aux SCVs une adhésion tissulaire accrue et une plus grande propension à former des biofilms. L'implication des FnBPs dans l'internalisation des SCVs pourrait être plus clairement confirmée en générant et utilisant une souche SCV dont les deux gènes encodant les FnBPs ont été interrompus. Il serait aussi intéressant d'utiliser la spectroscopie de force afin de pouvoir étudier directement l'interaction entre *S. aureus* et la cellule hôte. Il serait ainsi possible de suivre en temps réel l'internalisation de bactéries en absence ou présence de FnBPs en mesurant la déflexion d'un cantilevier décoré de bactéries (sauvages ou mutantes) en fonction du temps lors d'un contact avec la cellule hôte (Cuerrier *et al.*, 2009). Il est aussi envisageable que les voies de signalisation impliquées ou activées dans la cellule lors de l'internalisation de *S. aureus* pourraient être étudiées à l'aide de cette technique. Les avenues possibles sont ici innombrables surtout lorsque nous considérons que les microscopes/spectroscopes de force atomique peuvent être conjugués aux techniques conventionnelles de microscopie (Lamontagne *et al.*, 2008).

Les résultats de mes études doctorales démontrent très clairement que SigB est un facteur des plus importants à la réplication ou survie des SCVs à l'intérieur des cellules épithéliales pulmonaires FK (voir Annexe 3, Figure 7). Étant donné qu'il a déjà été démontré que SigB influence l'induction de la mort cellulaire par les souches à la croissance normale (Haslinger-Loffler *et al.*, 2005; Kubica *et al.*, 2008), que la surexpression de Hla peut être impliquée dans l'induction de la mort de la cellule hôte (Haslinger *et al.*, 2003; Jarry *et al.*, 2008; Klein *et al.*, 2006; Kubica *et al.*, 2008) et que SigB module très clairement l'hémolyse et l'expression d'*hla* chez une souche SCV (voir Annexe 3, Figure 8), nous avons tout d'abord posé l'hypothèse que SigB permet aux SCVs de se répliquer/survivre dans la cellule hôte sans induire la mort de cette dernière, ce qui ne semble finalement pas être le cas (voir Annexe 3, Figure 8). En considérant que SigB est fortement impliqué dans la survie des SCVs en phase stationnaire (Annexe 1, Figure 8) et qu'il est connu pour contrôler la réponse à certains stress chez les souches normales (Chan *et al.*, 1998; Gertz *et al.*, 1999; Giachino *et al.*, 2001; Horsburgh *et al.*, 2002), il est possible que SigB influence l'expression de gènes directement impliqués dans la survie des SCVs à l'intérieur des divers compartiments de la cellule hôte. Plus précisément, il a été montré que SigB influence la résistance à des pH acides et à des stress oxydatifs (Chan *et al.*, 1998), deux stress potentiellement rencontrés dans la cellule hôte (*p. ex.* dans les phagolysosomes). Cependant, la localisation intracellulaire des SCVs reste indéterminée, bien que la répression du système *agr* et de Hla suggère que les SCVs sont bel et bien incapables d'accéder au cytosol de la cellule hôte (Giese *et al.*, 2009; Giese *et al.*, 2011; Jarry *et al.*, 2008; Kubica *et al.*, 2008). Il reste aussi à savoir si la localisation intracellulaire des SCVs est identique chez les cellules épithéliales non-FK et FK (Jarry et Cheung, 2006). Cela pourrait être effectué en utilisant des anticorps dirigés contre les marqueurs d'endosomes LAMP-1 et LAMP-2, qui sont respectivement présents dans les membranes des endosomes tardifs et des lysosomes, et la V-ATPase, une protéine permettant d'acidifier les vésicules endosomales (Jarry et Cheung, 2006; Jarry *et al.*, 2008). Alternativement, des lignées cellulaires exprimant YFP-Fc pourraient être utilisées afin de suivre la sortie du *S. aureus* des phagolysosomes (Giese *et al.*, 2011). Il serait aussi intéressant d'évaluer la capacité des SCVs à survivre dans les phagocytes

professionnels en infectant des macrophages avec ces variants et en évaluant le nombre de CFU intracellulaires en fonction du temps (Kubica *et al.*, 2008).

Nos résultats confirment que la destinée du *S. aureus* intracellulaire est influencée par la fonctionnalité du CFTR chez les cellules épithéliales (Chapitre 4) (Jarry et Cheung, 2006). Ainsi, nous avons démontré que les infections intracellulaires à *S. aureus* suivent des cinétiques différentes dans les cellules FK et que cela se traduit entre autres par des niveaux supérieurs d'infection à 24 heures post-invasion. Par la suite, les niveaux d'infection redeviennent similaires entre les cellules non-FK et FK, les souches normales disparaissant presque complètement tandis que les SCVs s'accumulent de plus en plus à l'intérieur des cellules indépendamment de la fonctionnalité du CFTR. Une des hypothèses expliquant la propension des voies respiratoires FK à être infectées par des bactéries stipule que la colonisation bactérienne des voies respiratoires est facilitée par l'état pro-inflammatoire induit par une activité NFκB accrue (Hunter *et al.*, 2010; Joseph *et al.*, 2005; Rubin, 2007; Tirouvanziam *et al.*, 2000; Vij *et al.*, 2009). Étant donnée la possibilité que l'activité NF-κB influence l'interaction entre le *S. aureus* et la cellule hôte (Oviedo-Boyso *et al.*, 2008; Ratner *et al.*, 2001; Wang *et al.*, 2010), nous avons testé l'hypothèse que l'influence du CFTR sur les infections intracellulaires à *S. aureus* implique ce facteur (Chapitre 4). Il semble en effet que l'interaction entre le CFTR et NF-κB influence le niveau d'infection intracellulaire à *S. aureus*, mais le mécanisme complet reste à élucider. Une des possibilités est que l'activité de NF-κB inhibe l'induction de la mort des cellules épithéliales lors d'infections (Perkins, 2007; Wang *et al.*, 2010). Il serait pertinent de déterminer d'une façon plus globale les voies de signalisation activées par le *S. aureus* intracellulaire lors de l'infection de cellules FK ainsi que celles pouvant promouvoir ou inhiber l'infection. Mes travaux suggèrent que l'état pro-inflammatoire des poumons FK favorise les infections intracellulaires à *S. aureus* et, par conséquent, pourrait partiellement expliquer la récurrence des infections à *S. aureus*. Mes travaux supportent aussi l'idée que l'utilisation d'anti-inflammatoires pourrait contrer les infections chroniques à *S. aureus* chez les patients FK.

La capacité des SCVs de *S. aureus* à causer des infections chroniques n'a toujours pas été clairement démontrée dans un modèle d'infection expérimentale et il n'est pas certain que ce phénotype confère un avantage à la bactérie *in vivo*. Il semble tout de même que ce phénotype puisse permettre une plus grande persistance malgré les traitements antibiotiques dans deux différents modèles d'infections (Bates *et al.*, 2003; Brouillette *et al.*, 2004), mais il ne peut pas être conclu que cette persistance est causée par la formation de biofilm, la persistance intracellulaire ou tout simplement le métabolisme plus lent de la bactérie. Aucune étude à propos des facteurs de virulence importants aux SCVs *in vivo* n'a été publiée à ce jour. Il a récemment été montré que le phénotype SCV émerge d'infections prolongées (Tuchscherr *et al.*, 2011), mais cette étude ne permet pas de conclure des avantages dont les SCVs jouissent au cours des infections ou des facteurs de virulence importants à leur pathogenèse. Au cours de mes études doctorales, j'ai démontré que SigB est important à l'émergence de SCVs *in vivo* lors d'une exposition aux aminoglycosides en utilisant un modèle de mammite murine (Chapitre 2). J'ai aussi montré dans un modèle d'infection pulmonaire que les SCVs sont favorisés lors d'infections combinées avec des bactéries normales et que SigB (mais pas FnBPA) est important à ce phénomène (voir Annexe 3, Figure 9). Il est intéressant de constater que le phénotype SCV semble prendre de l'importance dans un contexte d'infection où des bactéries au phénotype normal sont présentes puisque les SCVs sont rarement trouvés seul au cours des infections (Proctor *et al.*, 2006). Ceci constitue les premières démonstrations que SigB à une importance réelle à la pathogenèse des SCVs.

Il serait particulièrement intéressant d'analyser l'importance de SigB dans la co-infection de souches SCVs avec *P. aeruginosa* puisque le phénotype SCV permet à *S. aureus* de mieux survivre lorsqu'en présence de *P. aeruginosa* (Biswas *et al.*, 2009). Le modèle de mammite murine avec la pression de la tétée pourrait aussi être utilisé pour confirmer le rôle des FnBPs dans la pathogenèse des SCVs. Mais, par-dessus tout, il serait bien d'utiliser des modèles expérimentaux d'infections pulmonaires chroniques ou mimant les conditions retrouvées chez le patient FK (Kukavica-Ibrulj et Levesque, 2008) afin d'étudier la pathogenèse des SCVs. Il

est connu que les différentes lignées de souris n'ont pas la même susceptibilité aux infections à *S. aureus* (von Kockritz-Blickwede *et al.*, 2008) et les souris CD-1 utilisées pendant mon doctorat éliminent les infections pulmonaires à *S. aureus* avec le temps. Il serait intéressant d'utiliser une lignée hautement susceptible aux infections pulmonaires à *S. aureus* afin de vérifier plus facilement l'hypothèse que les infections aux SCVs sont associées à des symptômes moins fulgurants.

Il a été démontré que l'invasion des cellules non phagocytaires par les SCVs n'est pas accompagnée par l'activation d'une réponse immunitaire prononcée et que l'apparition de SCVs *in vivo* est associée à une diminution de la réponse pro-inflammatoire dans des modèles d'infections à long terme (Chapitre 5) (Tuchscherr *et al.*, 2010; Tuchscherr *et al.*, 2011), offrant une explication à comment les SCVs peuvent persister chez l'hôte d'une façon asymptomatique. Cependant, bien que le système *agr* joue un rôle dans l'activation du système immunitaire lors de l'invasion de cellules non phagocytaires par des souches normales (Grundmeier *et al.*, 2010), les facteurs qui, par leur absence ou présence, expliquent cette moins grande stimulation du système immunitaire par les SCVs restent inconnus. Il sera important de mener des études au niveau de la réaction immunitaire de cellules ou d'organismes mimant les conditions FK lors d'infection à *S. aureus* étant donné l'état particulier du système immunitaire dans cette pathologie. Si *S. aureus* doit bel et bien être considéré comme un pathogène intracellulaire facultatif (Sendi et Proctor, 2009), il pourrait être bénéfique de développer des vaccins dirigés vers une immunité à médiation cellulaire afin de pouvoir éliminer efficacement les infections chroniques à *S. aureus*. L'utilisation de nouveaux adjuvants, tels que les dinucléotides cycliques, pourrait permettre de donner cette direction à la réponse immunitaire (Chen *et al.*, 2010; Ebensen *et al.*, 2010; Karaolis *et al.*, 2007).

Comme déjà souligné, la résistance des bactéries pathogènes aux antibiotiques est un problème majeur auquel l'humanité doit remédier rapidement. En plus de la multirésistance aux antibiotiques rencontrée chez plusieurs souches de staphylocoques (Witte *et al.*, 2008), *S. aureus* est capable d'adopter le phénotype SCV et d'ainsi se protéger de l'action des antibiotiques en se cachant dans les cellules de l'hôte et en formant du biofilm. De plus, les SCVs sont moins susceptibles à certains antibiotiques, dont ceux de la classe des aminoglycosides. Il est possible que les SCVs constituent un phénotype tolérant à plusieurs autres antibiotiques tel que décrit chez les cellules dormantes/persistantes (Lewis, 2007) et que ce facteur soit méconnu à cause de l'utilisation de techniques inappropriées à la caractérisation de la tolérance aux antibiotiques. La tolérance aux antibiotiques est l'habileté d'un microorganisme à ne pas être tué par l'activité d'un antibiotique et est différente de la résistance aux antibiotiques d'un point de vue mécanistique puisqu'elle est attribuable à l'acquisition transitoire d'un état de dormance ou de croissance très lente (Lewis, 2007). Étant donné que le métabolisme des SCVs est considérablement plus lent que celui des souches normales, il est possible que ces variants soient moins susceptibles à l'effet bactéricide de certains composés. Bien que la susceptibilité des SCVs aux antibiotiques ait été étudiée d'une façon assez exhaustive à l'aide de protocoles déterminants les concentrations minimales inhibitrices, il serait pertinent d'évaluer l'effet d'une collection d'antibiotiques sur les SCVs en utilisant un protocole de courbes de bactéricidie (voir Chapitre 6 et 7) et ainsi déterminer la tolérance aux antibiotiques des SCVs. L'utilisation d'antibiothérapies prenant en compte le phénotype SCVs aidera vraisemblablement à combattre les infections chroniques à *S. aureus*.

La tomatidine a plusieurs des caractéristiques requises à une molécule afin de contrer les infections chroniques à *S. aureus*. Elle a une activité bactériostatique contre les SCVs et a la capacité d'inhiber la réplication intracellulaire de ces souches variantes (Chapitre 6). La tomatidine a aussi la capacité d'augmenter l'efficacité des aminoglycosides contre les souches normales et les souches MRSA en plus d'inhiber l'activité de leur système *agr* (Chapitre 7). Il semble donc que les propriétés de la tomatidine pourraient aider à l'élimination des SCVs

intracellulaires tout en contrant les infections aiguës à *S. aureus* lors d'une utilisation combinée avec les aminoglycosides. Cependant, un des majeurs problèmes de la tomatidine est sa faible solubilité, ce qui nous a jusqu'à maintenant empêchés de vérifier l'efficacité de cette molécule dans les différents modèles d'infections expérimentaux utilisés au laboratoire du Pr François Malouin et qui constitue immanquablement un obstacle à l'utilisation de cette molécule en clinique. Actuellement, le Pr Éric Marsault et son équipe synthétisent des analogues structuraux afin d'optimiser les propriétés de cette molécule. Nous espérons obtenir un composé cliniquement utilisable dans le combat des infections pulmonaires chroniques à *S. aureus* rencontrées chez les patients FK.

CONCLUSION

Mes travaux de recherche suggèrent que le phénotype SCV confère un avantage à *S. aureus* lors d'infections pulmonaires chroniques chez les patients FK et que la virulence de ces souches variantes est fortement influencée par le facteur sigma alternatif SigB. Plus spécifiquement, il a été montré que l'activité SigB des SCVs est constitutive et soutient l'expression de FnBPA, ce qui mène à une forte adhésion aux tissus de l'hôte et à la formation de biofilm. Il a aussi été montré que SigB permet aux SCVs de se répliquer et de survivre dans les cellules épithéliales pulmonaires FK. Finalement, les SCVs arborant un gène SigB fonctionnel semblent être favorisés lors d'infections pulmonaires en présence de bactéries au phénotype normal. Il serait intéressant d'étudier SigB en tant que cible thérapeutique comme il a été fait chez *Listeria monocytogenes*. En effet, il a récemment été montré que le fluoro-phényle-styrène-sulfonamide est un inhibiteur de l'activité SigB chez cette bactérie, mais aussi chez *B. subtilis*, suggérant que ce composé pourrait être actif contre plusieurs bactéries pathogènes Gram-positif (Palmer *et al.*, 2011).

Il est connu depuis longtemps que certains facteurs environnementaux favorisent l'émergence de SCVs. Cependant, bien que plusieurs de ces facteurs environnementaux puissent être retrouvés dans les poumons des patients FK, aucune association clinique entre ces facteurs et la présence des SCVs n'a encore été établie, surtout pour les souches déficientes dans la chaîne de transport des électrons. Mes travaux de recherche suggèrent que la formation de SCVs est un phénomène régulé et que des facteurs de transcription (*p. ex.* SigB) peuvent intervenir dans l'émergence de ce phénotype. Cependant, le mécanisme complet sous-jacent à cette régulation est loin d'être élucidé et devrait faire l'objet de travaux supplémentaires. En effet, plusieurs possibilités peuvent ici être explorées : l'effet de SigB sur la transposition de séquences d'insertion, le contrôle du temps de demi-vie d'ARN spécifiques, la fréquence de

l'inversion flip-flop, l'activation de systèmes toxine-antitoxine et le contrôle de modifications épigénétiques.

L'étude des interactions microbiennes dans le cadre d'infections impliquant simultanément des pathogènes de plusieurs espèces est une voie de recherche excitante et prometteuse. Plusieurs questions pourront être étudiées concernant le rôle des interactions microbiennes interspécifiques dans le contexte de la FK : quels sont les mécanismes moléculaires et génétiques impliqués dans la coexistence des différentes espèces bactériennes colonisant les voies respiratoires FK? Quel est l'impact de l'interaction entre ces pathogènes *in vivo* et sur la progression de la maladie? Il est possible que l'interférence ou la modulation de ces interactions microbiennes puissent constituer une nouvelle approche thérapeutique (voir Annexe 1).

La tomatidine a des propriétés très prometteuses dans le contexte du traitement des infections chroniques à *S. aureus* : inhibition de la réplication des SCVs en bouillon et dans les cellules hôte, action en synergie avec les antibiotiques de la classe des aminoglycosides et altération de la virulence des souches à la croissance normale. Cependant, beaucoup de questions fondamentales restent encore sans réponses au sujet du mode ou des modes d'action de la tomatidine et de son efficacité *in vivo*. De plus, certaines des propriétés de cette molécule rendent sont utilisation clinique difficilement envisageable. Il reste à souhaiter que la synthèse d'analogues structuraux débouche sur un composé aux propriétés améliorées.

L'élucidation des mécanismes moléculaires déterminant les différentes propriétés bactériennes impliquées dans la persistance des infections pourrait avoir un impact autant biomédical que fondamental. Il est probable que l'élaboration d'une thérapie ciblant un ou plusieurs des

facteurs contribuant à l'établissement d'infections pulmonaires chroniques chez les patients FK augmentera l'efficacité des traitements actuels.

ANNEXE 1

Outcome and prevention of *Pseudomonas aeruginosa-Staphylococcus aureus* interactions during pulmonary infections in cystic fibrosis

RÉSUMÉ : Les patients FK succombent fréquemment à des complications cliniques subséquentes aux infections bactériennes chroniques. Il devient évident que plusieurs des espèces bactériennes retrouvées dans les voies respiratoires FK interagissent afin d'influencer la progression de la maladie. *P. aeruginosa* et *S. aureus* sont communément, et souvent simultanément, retrouvés chez les patients FK. Des études ont souligné l'influence de *P. aeruginosa* sur la virulence de *S. aureus* et ont révélé que certains exoproduits de *P. aeruginosa* provoquent l'émergence de SCVs chez *S. aureus*. En comparaison aux souches prototypes, les SCVs produisent moins de toxines et d'autres exoproduits impliqués dans la destruction des tissus et le déclenchement de la réponse immunitaire de l'hôte. À l'inverse, les SCVs expriment des adhésines, produisent du biofilm et sont persistants dans les cellules non phagocytaires. L'émergence de SCVs pourrait contribuer au développement d'infections chroniques en protégeant la bactérie contre le système immunitaire et l'action de certains antibiotiques. Les efforts actuels sont dirigés vers le développement de nouvelles approches thérapeutiques interférant avec les interactions bactériennes interspécifiques et les processus pathogéniques qui sont impliqués dans la persistance des infections pulmonaires FK.

RÉFÉRENCE: Mitchell, G., and Malouin, F. (2012) Outcome and prevention of *Pseudomonas aeruginosa-Staphylococcus aureus* interactions during pulmonary infections in cystic fibrosis. In Cystic Fibrosis - Renewed Hopes Through Research, D. Sriramulu, ed. (Rijeka, Croatia: InTech), pp. 181-206. Page web: http://www.intechopen.com/books/cystic-fibrosis-renewed-hopes-through-research/outcome-and-prevention-of-pseudomonas-aeruginosa-staphylococcus-aureus-interactions-during-pulmonary.

ANNEXE 2

Résumés des contributions supplémentaires

1- *Staphylococcus aureus* and biofilm-forming small-colony variants from airways of adult cystic fibrosis patients

RÉSUMÉ/ABSTRACT : *Staphylococcus aureus* is a predominant pathogen in cystic fibrosis (CF) but little is known about its presence in the adult population. Here, we evaluated the prevalence and characteristics of *S. aureus* among 32 adult CF patients over a three-year study. Isolates were characterized by their genotype, toxins profiles, antibiotic susceptibility profiles, SCCmec types, biofilm production and persistence. From the 18 (56%) patients that were positive for *S. aureus*, eight were positive for small-colony variants. Presence of *S. aureus* was affected by patient's antibiotic treatment and presence of *P. aeruginosa*. Overall, 78% of *S. aureus*-positive patients have been colonized with *S. aureus* showing resistance to at least one of tested antibiotics. Although isolated SCVs produced more biofilm than prototypical *S. aureus* ($P < 0.05$), neither had an impact on the patient forced expired volume in 1 second comparatively to *P. aeruginosa* ($P < 0.05$). Results suggest that *S. aureus* isolates played a significant role in persistent infections in adult CF patients.

RÉFÉRENCE : Séguin, D.L., Lafrance, M., **Mitchell, G.**, Rodrigue, S., Frost, E.H., Michaud, S., Cantin, A.M., et Malouin, F. *Staphylococcus aureus* and biofilm-forming small-colony variants from airways of adult cystic fibrosis patients. En préparation pour soumission.

2- Interspecific molecular interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* retrieved from adult cystic fibrosis patients

RÉSUMÉ/ABSTRACT: *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) are the most prevalent pathogens in cystic fibrosis (CF) associated airway infections. It is suspected that interspecific molecular interactions can modulate virulence and pathophysiology. This study aims to characterize the influence of PA exoproducts on SA growth and biofilm formation. 65 clinical PA isolates were obtained from the respiratory tract of 32 adult CF patients. From those, some were co-isolated with SA and a total of 31 SA-PA pairs were collected. PA strains were grown for 20 h and culture supernatants were analyzed by LC/MS for detection and dosage of quorum sensing-related compounds. PA culture supernatants were also used to supplement, in a dose-dependent manner, SA cultures to measure their impact on growth and biofilm production. Supernatants from PA showed a wide range of growth-inhibitory and biofilm-stimulatory activities against a control SA strain. PA supernatants able to reduce SA growth were more efficient in stimulating biofilm production by SA. The extent of biofilm formation by SA exposed to PA supernatants was positively correlated to the level of 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) and *Pseudomonas* Quinolone Signal (PQS) production by PA isolates. Also, supernatants of PA14-isogenic mutants deficient in PQS and HQNO production significantly stimulated less biofilm formation than that seen with wild type PA14. Interestingly, when tested in pairs, supernatants from PA isolates did not stimulate much biofilm production by the co-isolated SA strains compared to the stimulatory effect observed on the control SA strain. Accordingly, some of the clinical SA isolates were not stimulated by strain PA14 despite adequate production of HQNO. Although HQNO and PQS can inhibit growth and stimulate biofilm formation of a control SA in a dose-dependent manner, results obtained from co-isolated PA-SA pairs did not follow this trend and suggests that co-existence requires specific adaptations by either counterpart. Overall, this study gives insights on microbial interactions occurring in the establishment of polymicrobial infections in CF.

RÉFÉRENCE: Fugère, A.*, Séguin, D.L.*, Brouillette, E, **Mitchell, G.**, Boulanger, S., Déziel, É., Frost, E.H., and Malouin, F. Interspeccific molecular interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* retrieved from adult cystic fibrosis patients. En préparation pour soumission.

*Alexandre Fugère et David Lalonde Séguin ont contribués également à cette étude.

3- Steroid alkaloids and uses thereof as antimicrobial agents against electron transport-deficient microbes and as potentiators for antimicrobial agents against pathogenic bacteria

RÉSUMÉ/ABSTRACT: The present invention includes novel compounds and compositions comprising said compounds which exhibit antimicrobial activity against extracellular or intracellular electron transport-deficient microbes and/or increase the antimicrobial activity of aminoglycoside antibiotics against their targets, and which are useful as antibacterial agents for treatment or prophylaxis of monomicrobic or polymicrobic bacterial infections or for the reduction of antibiotic resistance development in animals or in humans, or for use as antiseptics or agents for sterilization or disinfection.

RÉFÉRENCE: Malouin, F., **Mitchell, G.**, Bouarab, K., Marsault, E., Chagnon, F., Boulanger, S., and Guay, I. Steroid alkaloids and uses thereof as antimicrobial agents against electron transport-deficient microbes and as potentiators for antimicrobial agents against pathogenic bacteria. International patent application # PCT/CA2012/050087. Déposé le 15 février 2012.

4- Transcriptional analysis of antibiotic resistance and virulence genes in multiresistant hospital-acquired MRSA

RÉSUMÉ/ABSTRACT: The staphylococcal chromosome cassette *mec* cannot solely explain the multiresistance phenotype or the relatively mild virulence profile of hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA). This study reports that several multiresistant HA-MRSA strains differently expressed genes that may support antibiotic resistance, modify the bacterial surface and influence the pathogenic process. Genes encoding efflux pumps (*norA, arsB, emrB*) and the macrolide resistance gene *ermA* were found to be commonly expressed by HA-MRSA strains, but not in the archetypal MRSA strain COL. At equivalent cell density, the *agr* system was considerably less activated in all MRSA strains (including COL) in comparison with a prototypic antibiotic-susceptible strain. These results are in contrast to those observed in recent community-acquired MRSA isolates and may partly explain how multiresistant HA-MRSA persist in the hospital setting.

RÉFÉRENCE: Pruneau, M., **Mitchell, G.**, Moisan, H., Dumont-Blanchette, E., Jacob, C.L., and Malouin, F. (2011). Transcriptional analysis of antibiotic resistance and virulence genes in multiresistant hospital-acquired MRSA. FEMS Immunol. Med. Microbiol. *63*, 54-64.

5- Plant antimicrobial agents and their effects on plant and human pathogens

RÉSUMÉ/ABSTRACT: To protect themselves, plants accumulate an armoury of antimicrobial secondary metabolites. Some metabolites represent constitutive chemical barriers to microbial attack (phytoanticipins) and others inducible antimicrobials (phytoalexins). They are extensively studied as promising plant and human disease-controlling agents. This review discusses the bioactivity of several phytoalexins and phytoanticipins

defending plants against fungal and bacterial aggressors and those with antibacterial activities against pathogens affecting humans such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* involved in respiratory infections of cystic fibrosis patients. The utility of plant products as "antibiotic potentiators" and "virulence attenuators" is also described as well as some biotechnological applications in phytoprotection.

RÉFÉRENCE: González-Lamothe R., **Michell, G.**, Gattuso, M., Diarra, M.S., Malouin, F., and Bouarab, K. (2009). Plant antimicrobial agents and their effects on plant and human pathogens. Int. J. Mol. Sci. *10*, 3400-3419.

6- Single-molecule dynamic force spectroscopy of the fibronectin-heparin interaction

RÉSUMÉ/ABSTRACT: The integrity of cohesive tissues strongly depends on the presence of the extracellular matrix, which provides support and anchorage for cells. The fibronectin protein and the heparin-like glycosaminoglycans are key components of this dynamic structural network. In this report, atomic force spectroscopy was used to gain insight into the compliance and the resistance of the fibronectin-heparin interaction. We found that this interaction can be described by an energetic barrier width of 3.1+/-0.2A and an off-rate of 0.2+/-0.1s^{-1}. These dissociation parameters are similar to those of other carbohydrate-protein interactions and to off-rate values reported for more complex interactions between cells and extracellular matrix components. Our results indicate that the function of the fibronectin-heparin interaction is supported by its capacity to sustain significant deformations and considerable external mechanical forces.

RÉFÉRENCE: **Mitchell, G.**, Lamontagne, C.A., Lebel, R., Grandbois, M., and Malouin, F. (2007). Single-molecule dynamic force spectroscopy of the fibronectin-heparin interaction. Biochem. Biophys. Res. Commun. *364*, 595-600.

ANNEXE 3

Résultats supplémentaires

Les résultats de cette section sont une extension de ma thèse de doctorat et visent à mieux définir les mécanismes moléculaires par lesquels SigB influence la virulence des SCVs isolés des patients FK. Plus précisément, cette étude a les objectifs suivants :

1- Déterminer le(s) mécanisme(s) moléculaire(s) par lequel les SCVs forment une quantité accrue de biofilm.
2- Caractériser le(s) mécanisme(s) moléculaire(s) par lequel les SCVs persistent dans les cellules FK.
3- Caractériser le rôle(s) de SigB et de FnBPA dans la virulence des SCVs.

Ces résultats ont été obtenus en très grande majorité au cours de mes expériences au laboratoire. Par contre, certains autres résultats ont été générés par Karine Pépin Gaudreau au cours de sa maîtrise.

J'anticipe compléter et écrire un manuscrit en lien avec les résultats de cette annexe.

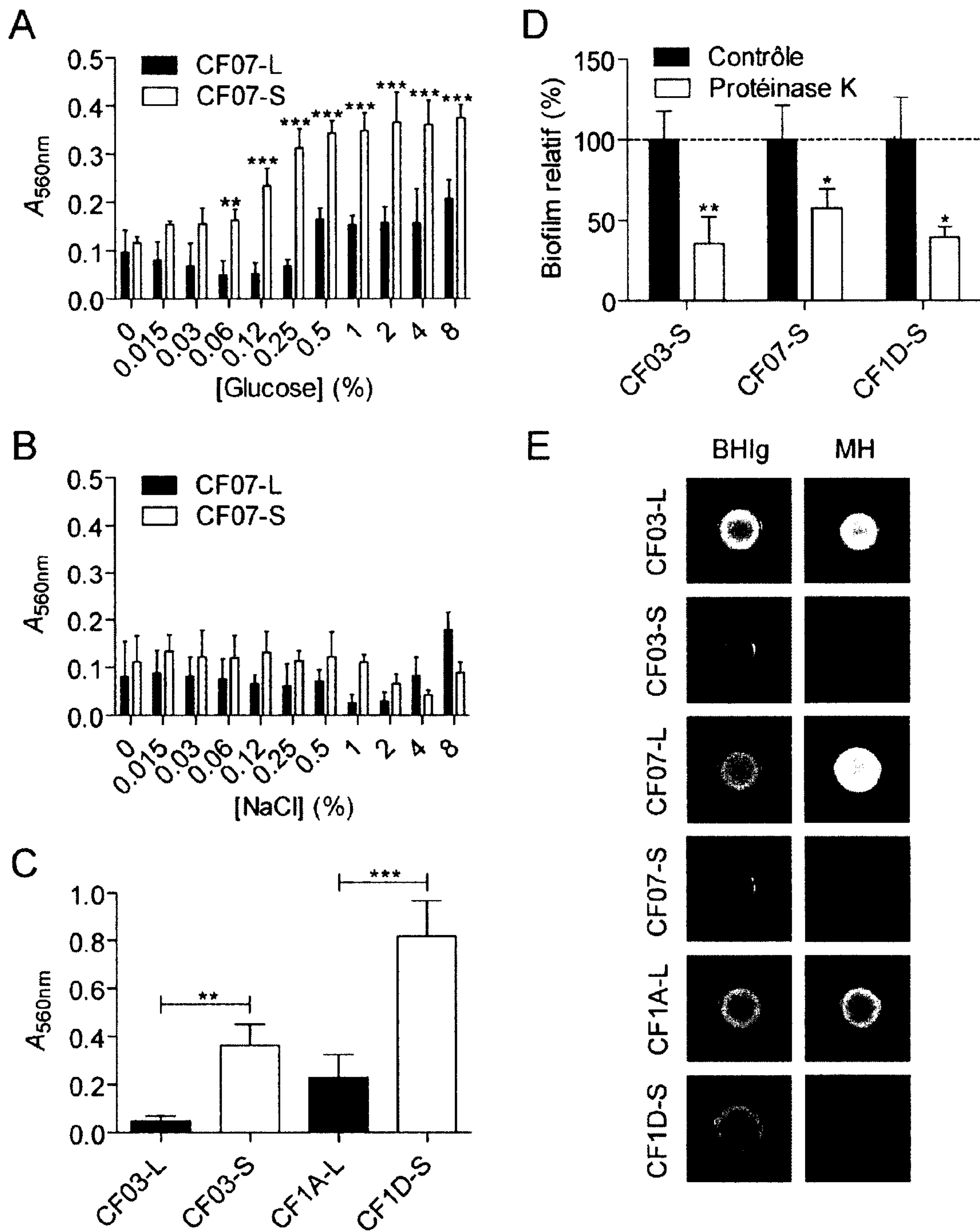


FIGURE 1. Les SCVs forment un biofilm dépendant des protéines et ont peu d'activité protéolytique. Formation de biofilm mesurée à A_{560nm} de la souche normale CF07-L et de la

souche SCV CF07-S en fonction de la concentration de glucose (A) et de NaCl (B) après 48 heures d'incubation. Les différences significatives entre CF07-L et CF07-S sont indiquées (**, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à deux facteurs suivi d'un post-test de Bonferonni (*n*=3). (C) Formation de biofilm de souches normales (CF03-L et CF1A-L) et de souches SCVs (CF03-S et CF1D-S) exposées à 0.25% de glucose pendant 48 heures. Les différences significatives entre les souches normales et SCVs sont indiquées (**, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par le test de student (*n*=3-4). (D) Essais de détachement à la protéinase K de biofilms formés par des souches SCVs (*n*=3). Les différences significatives entre les conditions contrôles et traitées sont indiquées (*, $P < 0.05$; **, $P < 0.01$) et ont été déterminées par le test de student. Les données ont été normalisées pour chacune des souches en condition contrôle. Les données sont représentées par des moyennes avec les déviations standards. (E) Évaluation de l'activité protéolytique de différentes souches normales et SCVs sur pétris-lait (BHI agar glucosé (BHIg) ou MH agar contenant du lait en poudre et incubés 24 et 48 heures, respectivement).

INTERPRÉTATION DES RÉSULTATS : Il a été démontré que l'exposition de *S. aureus* au glucose et au NaCl favorise la formation de biofilms dépendants des protéines et des polymères de N-acétyle-glucosamine, respectivement (O'Neill *et al.*, 2008). Les Figures 1A et 1B montrent que la formation de biofilm de la souche SCV CF07-S est significativement plus grande que celle de sa contrepartie normale lorsqu'en présence de glucose, ce qui suggère que les protéines extracellulaires jouent un rôle dans la formation de biofilm par les SCVs. La formation accrue de biofilm des SCVs lorsqu'en présence de glucose est montrée à la Figure 1C pour deux autres paires de co-isolats. Des essais de détachement à la protéinase K confirment que les protéines sont un des constituants importants des biofilms formés par les SCVs (Figure 1D). Il est connu que des mécanismes impliquant des protéases extracellulaires assurent la dispersion des biofilms dépendants des protéines (Boles et Horswill, 2008). La Figure 1E montre que l'activité protéolytique de trois souches SCVs est presque nulle et suggère que les mécanismes de dispersion du biofilm dépendants des protéases extracellulaires ne sont pas activés chez les SCVs.

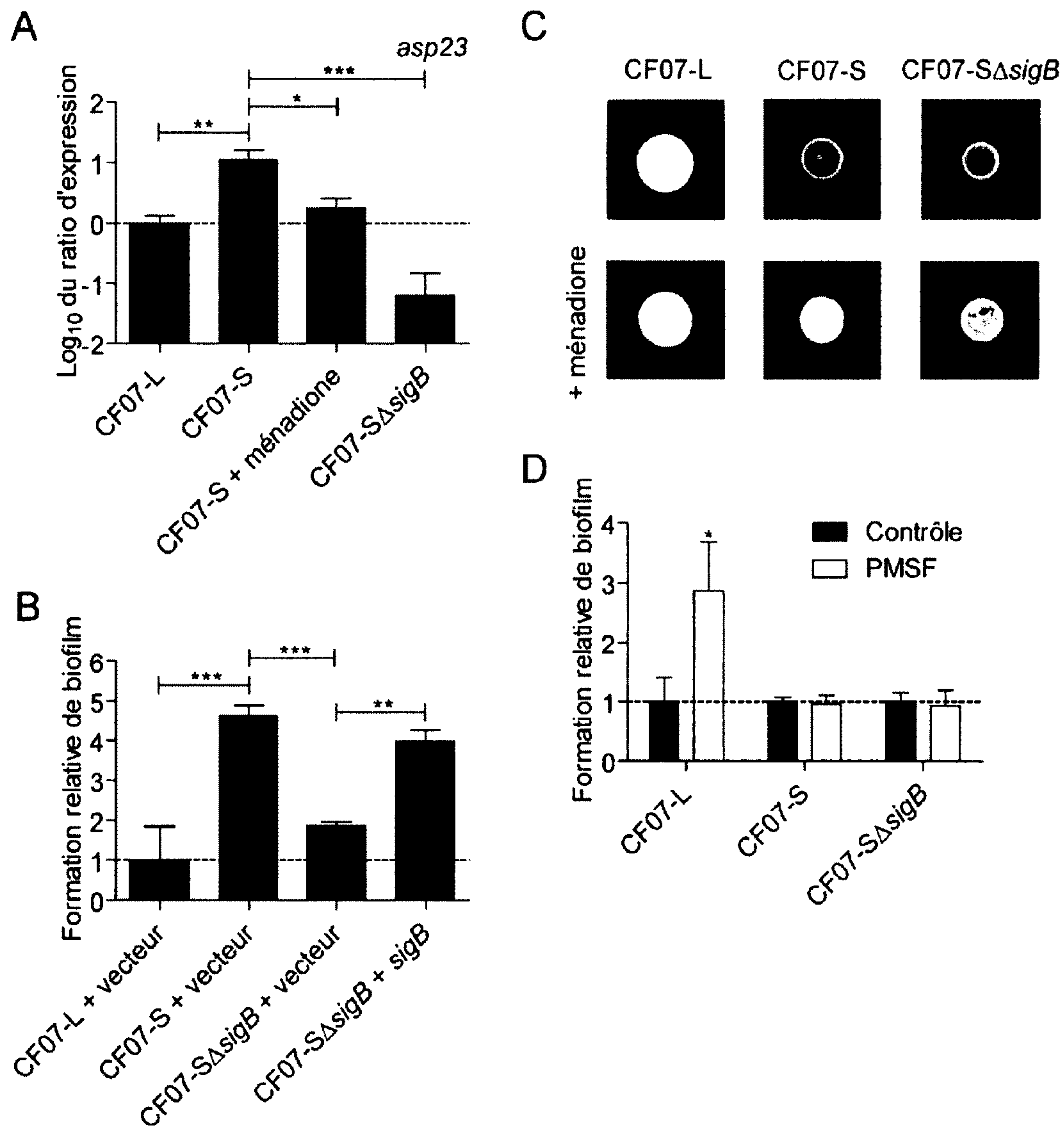


FIGURE 2. SigB module la formation de biofilm des SCVs sans influencer l'activité protéolytique. (A) Ratio d'expression du gène marqueur de l'activité SigB *asp23* en phase de croissance exponentielle précoce tel qu'évalué par PCR quantitative en temps réel. Les différences significatives par rapport à CF07-S sont indiquées (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à un facteur suivi d'un post-test de Dunnett (n=3-4). Les données sont exprimées par rapport à CF07-L. (B) Formation relative de biofilm

après 48 heures d'incubation en présence de $CdCl_2$ (0.25 µM) de CF07-L, CF07-S et CF07-SΔ*sigB* transportant un vecteur d'expression inductible vide ou contenant le gène *sigB*. Les différences significatives pertinentes sont indiquées (**, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à un facteur suivi d'un post-test de Tuckey (n=3). Les données sont exprimées par rapport à CF07-L transportant un vecteur vide. (C) Évaluation de l'activité protéolytique de CF07-L, CF07-S et CF07-SΔ*sigB* incubés 48 heures sur pétris-lait BHIg avec ou sans supplémentation à la ménadione. (D) Formation relative de biofilm de CF07-L, CF07-S et CF07-SΔ*sigB* en présence de l'inhibiteur de protéase PMSF (*phenylmethylsulfonyl fluoride*) pendant 48 heures d'incubation. Les différences significatives entre les conditions contrôles et traitées au PMSF sont indiquées (*, $P < 0.05$) et ont été déterminées par le test de student (n=3). Les données ont été normalisées pour chacune des souches en condition contrôle. Les données sont représentées par des moyennes avec les déviations standards.

INTERPRÉTATION DES RÉSULTATS : La Figure 2A confirme que l'activité SigB est accrue chez le SCV CF07-S en comparaison à sa contrepartie normale CF07-L et qu'elle est liée à la fonctionnalité de la chaîne de transport des électrons puisque la supplémentation métabolique de cette souche auxotrophe pour la ménadione rétablit un niveau d'expression normale d'*asp23*. La Figure 2B confirme que SigB est impliqué dans la formation d'un biofilm par les SCVs (voir Chapitre 2). Il a été démontré que SigB réprime l'activité protéolytique des souches normales et influence ainsi la dispersion des biofilms (Lauderdale *et al.*, 2009; Marti *et al.*, 2010). Cependant, la Figure 2C montre que l'activité protéolytique de CF07-S n'est pas activée par l'interruption génétique de *sigB*, bien qu'un effet de SigB sur l'activité protéolytique de cette souche soit observé lorsque sa croissance est supplémentée par la ménadione. Il a été montré que l'inhibiteur de protéases PMSF empêche les mutants *sigB* au phénotype normal de dégrader leur biofilm (Lauderdale *et al.*, 2009). La Figure 2D montre que le PMSF stimule bel et bien la formation (ou l'accumulation) de biofilm chez la souche normale, mais qu'il n'a pas d'effet sur la souche CF07-S et CF07-SΔ*sigB*. Les Figures 2C et 2D suggèrent donc que SigB n'est pas le facteur réprimant la dispersion des biofilms par les protéases extracellulaires chez les SCVs.

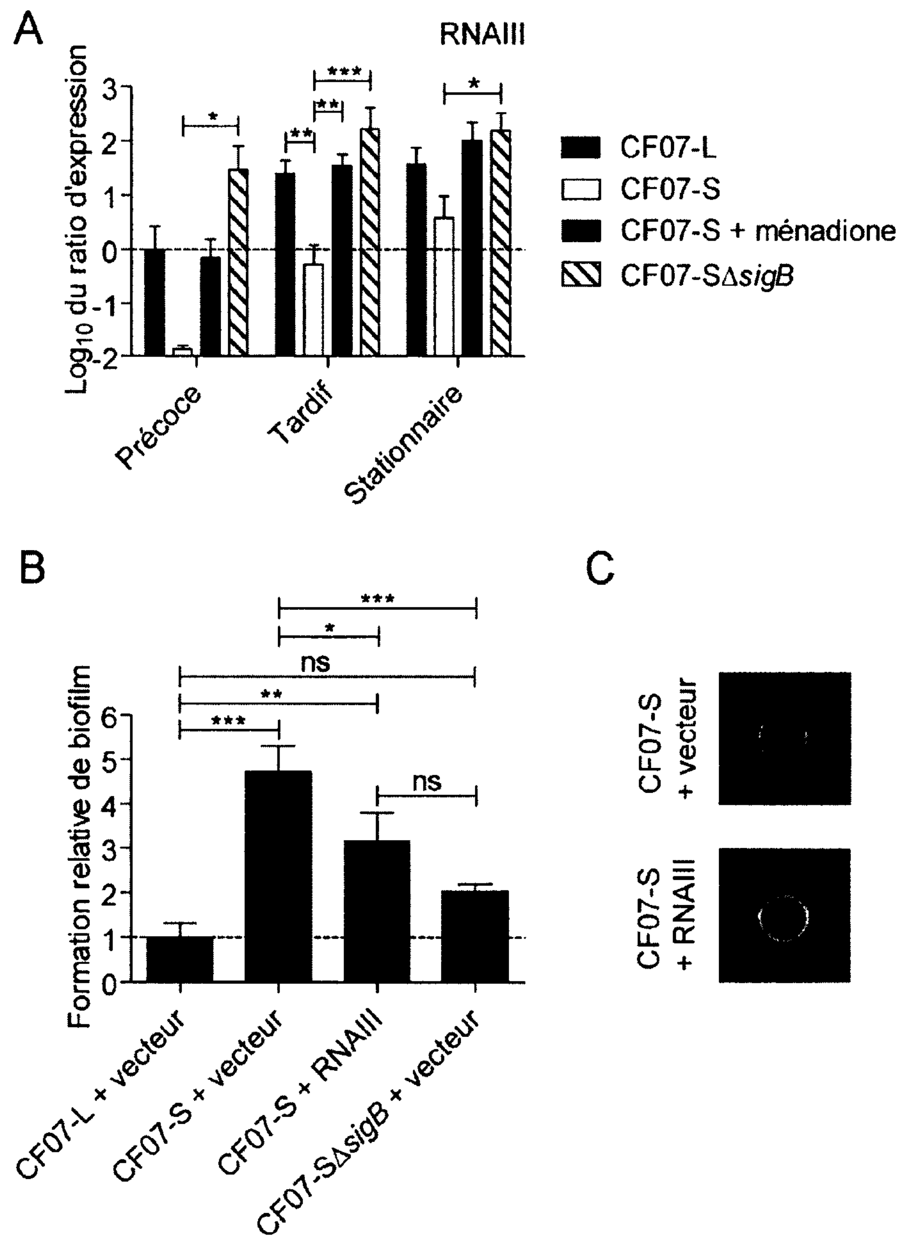


FIGURE 3. L'activité du système *agr* est modulée par SigB et influence la formation de biofilm sans moduler l'activité protéolytique chez les SCVs. (A) Cinétiques de ratio d'expression de RNAIII en fonction de la croissance tel qu'évalué par PCR quantitative en temps réel. Les différences significatives par rapport à CF07-S sont indiquées (*, $P < 0.05$; **,

$P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à un facteur suivi d'un post-test de Dunnett (n=3-4). Les données sont exprimées par rapport à CF07-L en phase de croissance exponentielle précoce. (B) Formation relative de biofilm après 48 heures d'incubation en présence de $CdCl_2$ (0.12 µM) de CF07-L, CF07-S et CF07-SΔ*sigB* transportant un vecteur d'expression inductible vide ou contenant RNAIII (n=3). Les différences significatives sont indiquées (ns, non-significatif; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à un facteur suivi d'un post-test de Tuckey. Les données sont exprimées par rapport à CF07-S transportant un vecteur vide. Les données sont représentées par des moyennes avec les déviations standards. (C) Évaluation de l'activité protéolytique de CF07-S transportant un vecteur d'expression inductible vide ou contenant RNAIII incubés 48 heures sur pétris-lait BHIg en présence de $CdCl_2$ (4 µM).

INTERPRÉTATION DES RÉSULTATS : Il est connu que l'activité du système *agr* influence la formation de biofilm en réprimant certains facteurs d'adhésion (Chan *et al.*, 2004) et en activant des mécanismes de dispersion (Beenken *et al.*, 2010; Boles et Horswill, 2008; Kong *et al.*, 2006; Lauderdale *et al.*, 2009). De plus, les SCVs répriment le système *agr* d'une façon dépendante de l'activité SigB (voir Chapitre 2). La Figure 3A montre que l'expression de RNAIII (l'effecteur du système *agr*) est retardée chez CF07-S en comparaison à la souche normale CF07-L et que ce retard d'expression est causé par l'état métabolique altéré du SCV. L'expression de RNAIII s'emballe drastiquement chez CF07-SΔ*sigB*, ce qui confirme que SigB réprime l'activité *agr* de CF07-S. Cependant, le rôle de SigB dans la formation de biofilm des SCVs implique possiblement une autre voie indépendante du système *agr* puisque la surexpression de RNAIII chez CF07-S n'abolit pas complètement la formation de biofilm (Figure 3B). La Figure 3C suggère que l'effet de la surexpression de RNAIII sur le biofilm de CF07-S n'est pas causé par les protéases extracellulaires.

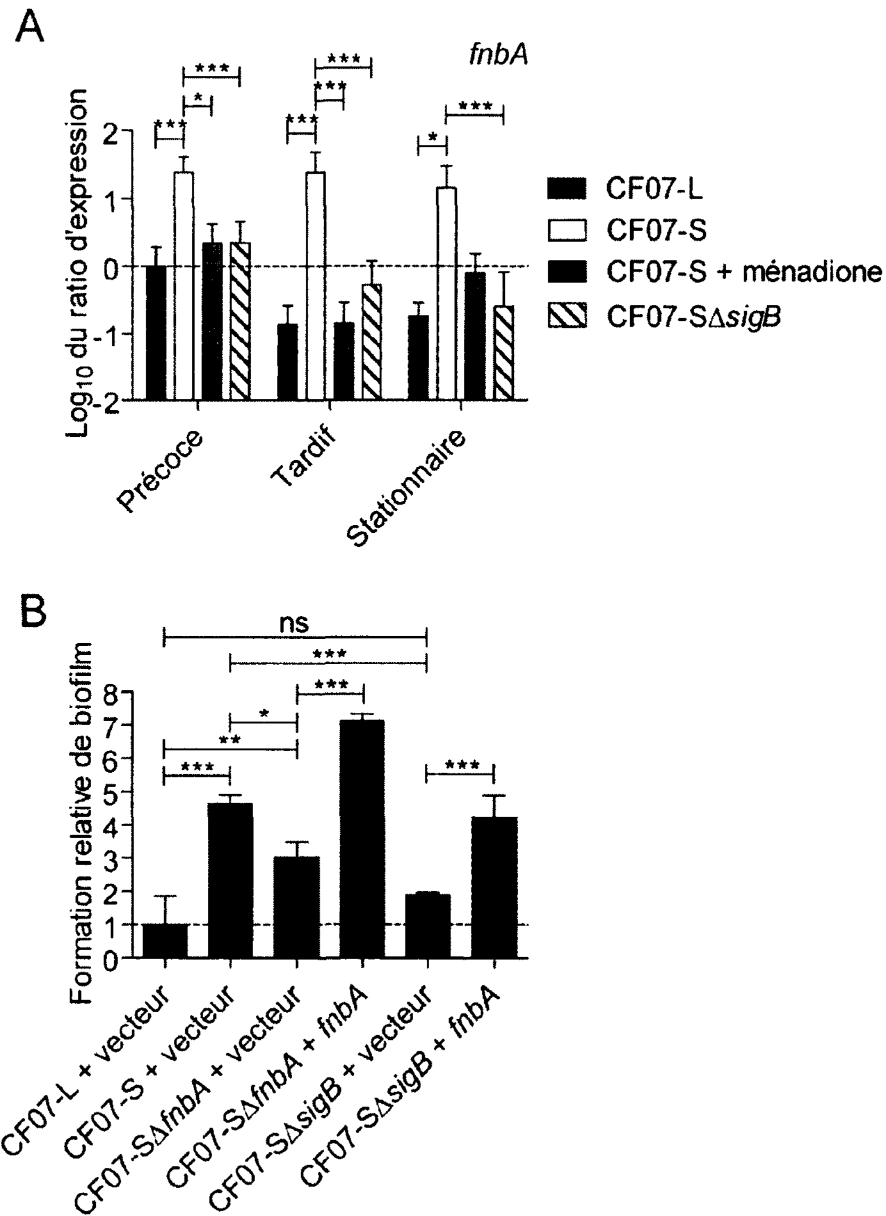
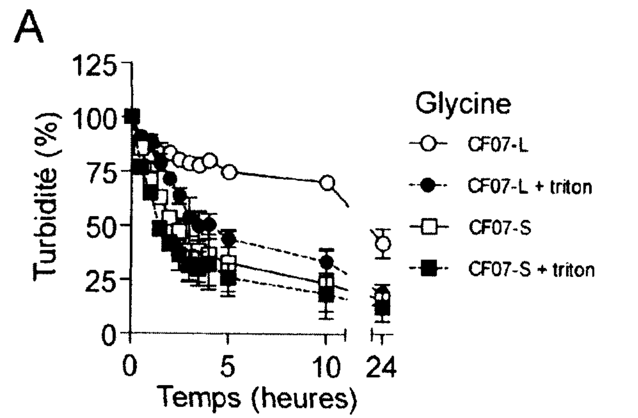
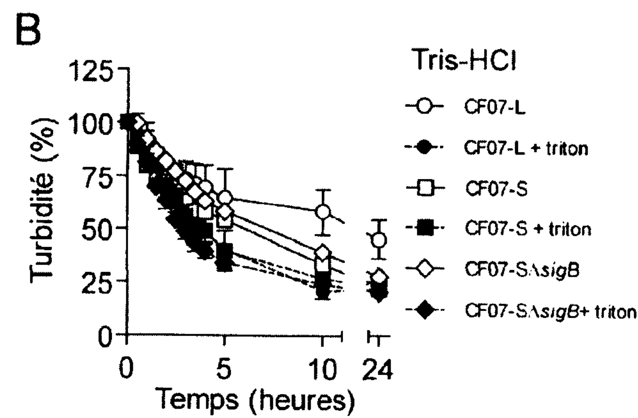


FIGURE 4. SigB module la formation de biofilm des SCVs en contrôlant entre autres l'expression de *fnbA*. (A) Cinétiques de ratio d'expression de *fnbA* en fonction de la croissance tel qu'évalué par PCR quantitative en temps réel. Les différences significatives par rapport à CF07-S sont indiquées (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par

l'ANOVA à un facteur suivi d'un post-test de Dunnett (n=2-8). Les données sont exprimées par rapport à CF07-L en phase de croissance exponentielle précoce. (B) Formation relative de biofilm après 48 heures d'incubation en présence de $CdCl_2$ (0.25 μM) de CF07-L, CF07-S, CF07-SΔ*fnbA* et CF07-SΔ*sigB* transportant un vecteur d'expression inductible vide ou contenant le gène *fnbA*. Les différences significatives pertinentes sont indiquées (ns, non-significatif; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à un facteur suivi d'un post-test de Tuckey. Les données sont exprimées par rapport à CF07-L transportant un vecteur vide (n=3). Les données sont représentées par des moyennes avec les déviations standards.

INTERPRÉTATION DES RÉSULTATS : L'activité SigB constitutive des SCVs entraîne une expression soutenue de *fnbA* au cours de toutes les phases de croissance (voir Chapitre 1). Il a été démontré que les FnBPs peuvent constituer la matrice des biofilms dépendants des protéines extracellulaires chez *S. aureus* (O'Neill *et al.*, 2008). La Figure 4A confirme l'expression accrue et soutenue de *fnbA* chez CF07-S en comparaison aux bactéries à la croissance normale et en comparaison à la souche CF07-SΔ*sigB*. FnBPA semble bel et bien impliqué dans la formation de biofilm des SCVs (Figure 4B). Cependant, la formation de biofilm de CF07-SΔ*fnbA* est encore supérieure à celle de CF07-L (Figure 4B), suggérant que d'autres facteurs jouent un rôle dans la formation de biofilm chez les SCVs.


A
Turbidité (%)
Temps (heures)
Glycine
CF07-L
CF07-L + triton
CF07-S
CF07-S + triton


B
Turbidité (%)
Temps (heures)
Tris-HCl
CF07-L
CF07-L + triton
CF07-S
CF07-S + triton
CF07-SΔsigB
CF07-SΔsigB+ triton

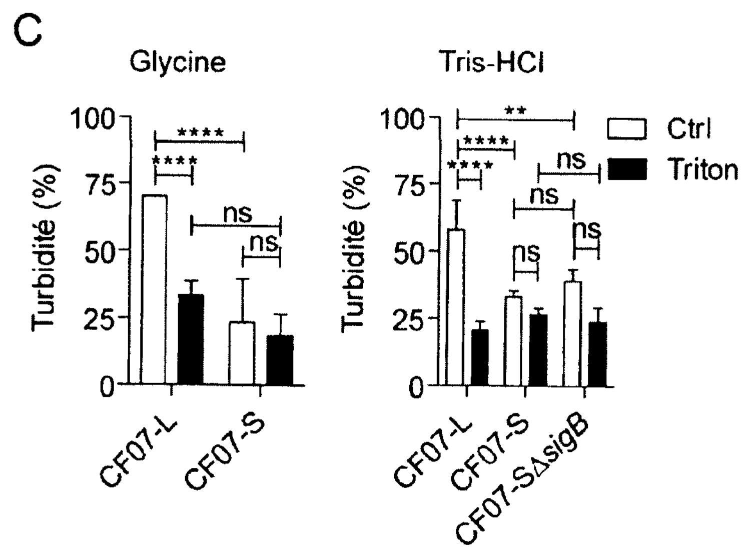

C
Glycine
Tris-HCl
Turbidité (%)
Ctrl
Triton
CF07-L
CF07-S
CF07-SΔsigB

FIGURE 5. L'activité autolytique des SCVs est plus grande que celle des souches normales, mais n'est pas contrôlée par SigB. Autolyse de CF07-L, CF07-S et CF07-SΔ*sigB* en fonction du temps en présence ou absence de triton dans un tampon glycine (A) ou Tris-HCl (B). Les résultats ont été analysés par l'ANOVA à deux facteurs suivi d'un post-test de Bonferroni et les différences significatives à 10 heures sont indiquées en (C) (ns, non-significatif; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) (n=2-4). Les résultats sont exprimés en pourcentage de la turbidité initiale pour chacune des conditions. Les données sont représentées par des moyennes avec les déviations standards.

INTERPRÉTATION DES RÉSULTATS : L'autolyse est une forme de mort cellulaire programmée qui contribue à la formation de biofilm par la libération d'ADN génomique extracellulaire servant alors de matrice (Houston *et al.*, 2011; Mann *et al.*, 2009; Rice *et al.*, 2007). Il a été démontré que l'autolyse est influencée par la force proton-motrice (et, par conséquent, l'activité de la chaîne de transport des électrons) (Patton *et al.*, 2006) ainsi que par l'activité de SigB (Patton *et al.*, 2005). La Figure 5 montre que l'autolyse de la souche SCV CF07-S est supérieure à celle de la souche normale CF07-L, mais que SigB n'est pas impliqué dans ce processus.

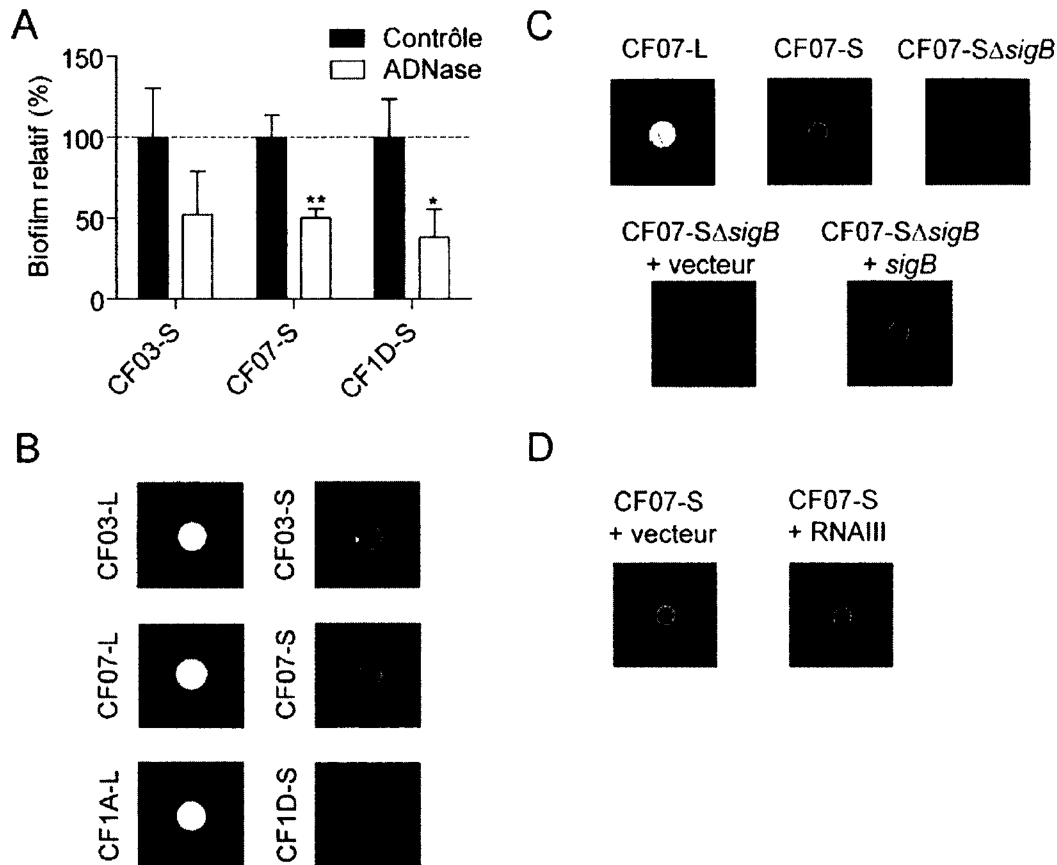


FIGURE 6. SigB régule l'activité nucléase des SCVs. (A) Essais de détachement à l'ADNase de biofilms formés par des souches SCVs (n=3). Les différences significatives entre les conditions contrôles et traitées sont indiquées (*, $P < 0.05$; **, $P < 0.01$) et ont été déterminées par le test de student (n=3). Les données ont été normalisées pour chacune des souches en condition contrôle et sont représentées par des moyennes avec les déviations standards. (B) Évaluation de l'activité nucléase de souches normales (CF03-L, CF07-L et CF1A-L) et SCVs (CF03-S, CF07-S et CF1D-S) incubées pendant 24 heures sur pétris-ADN. (C) Évaluation de l'activité ADNase de CF07-L, CF07-S, CF07-SΔ*sigB*, CF07-SΔ*sigB* + vecteur vide et CF07-SΔ*sigB* + vecteur *sigB* incubés pendant 48 heures sur pétris-ADN en présence de de $CdCl_2$ (0.25 μM). (D) Évaluation de l'activité protéolytique de CF07-S transportant un vecteur

d'expression inductible vide ou contenant RNAIII incubés 48 heures sur pétris-lait BHIg en présence de $CdCl_2$ (4 µM).

INTERPRÉTATION DES RÉSULTATS : Il a été démontré que la production de nucléases par les souches normales de *S. aureus* est contrôlée par SigB et qu'elle constitue un mécanisme de dispersion des biofilms (Kiedrowski *et al.*, 2011). La Figure 6A confirme que l'ADN génomique extracellulaire est un constituant important du biofilm des SCVs. La Figure 6B montre que les SCVs ont une activité nucléase diminuée comparativement aux souches normales. Cette activité nucléase est contrôlée par SigB, mais pas par le système *agr*, chez CF07-S. Ces résultats suggèrent que l'activité SigB des SCVs inhibe la production de nucléases et la dispersion des biofilms constitués d'ADN génomique extracellulaire.

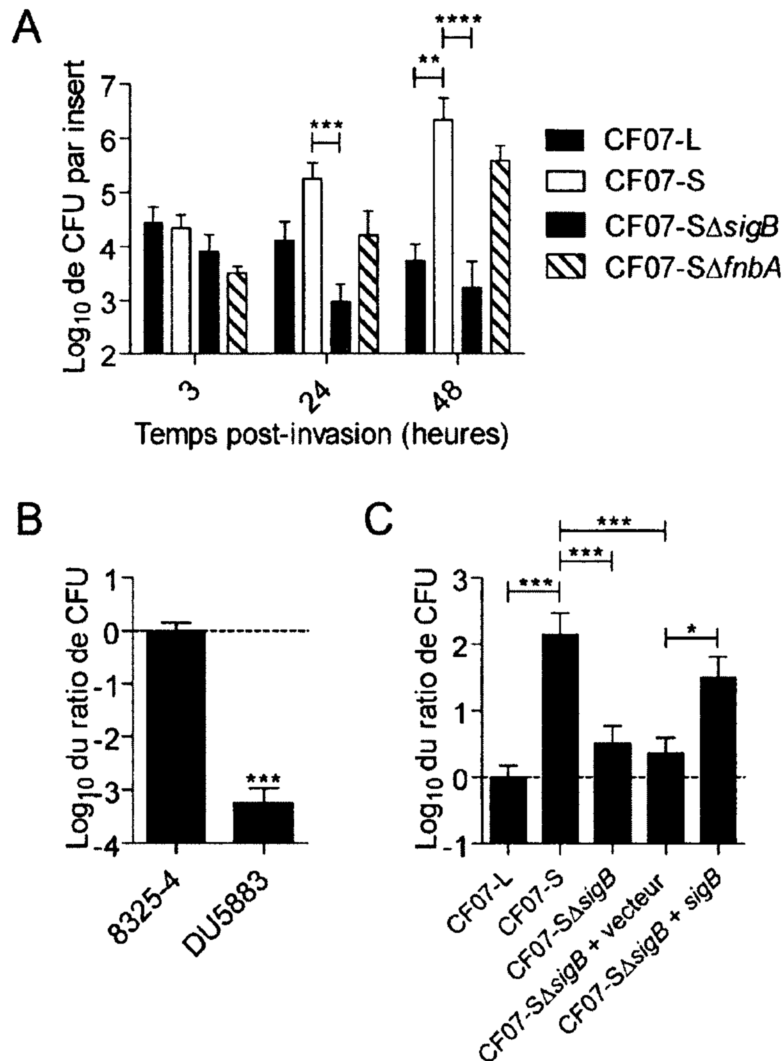


FIGURE 7. SigB est nécessaire à la persistance des SCVs dans des cellules épithéliales pulmonaires FK (Calu-3 shCFTR). (A) Cinétiques d'infection intracellulaire en CFU (*colony-forming unit*) par insert pour les souches CF07-L, CF07-S, CF07-SΔ*sigB* et CF07-SΔ*fnbA*. Les différences significatives sont indiquées (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) et ont été déterminées par l'ANOVA à deux facteurs suivi d'un post-test de Bonferonni (n=3-6). (B) Niveaux d'internalisation relatifs de 8325-4 et DU5883 (Δ*fnbAB*) déterminés à 3 heures post-invasion. La différence significative est indiquée (***, $P < 0.001$) et a été

déterminée par un test de student (n=3). Les données sont exprimées par rapport à 8325-4. (C) Niveaux relatifs d'infection de CF07-L, CF07-S, CF07-SΔ*sigB* ,CF07-SΔ*sigB* + vecteur vide + 0.25 µM $CdCl_2$ et CF07-SΔ*sigB* + *sigB* + 0.25 µM $CdCl_2$ à 48 heures post-invasion. Les différences significatives pertinentes sont indiquées (*, $P < 0.05$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à un facteur suivi d'un post-test de Tuckey (n=3-7). Les données sont exprimées par rapport à CF07-L et sont représentées par des moyennes avec les déviations standards.

INTERPRÉTATION DES RÉSULTATS : Il a été suggéré que SigB influence l'invasion cellulaire et la persistance intracellulaire des SCVs (Moisan *et al.*, 2006). La Figure 7A confirme que les SCVs ont la capacité de s'accumuler dans des cellules épithéliales pulmonaires FK. L'internalisation des SCVs ne semble pas influencée significativement par *fnbA*, suggérant que la seule présence de *fnbB* permet une internalisation efficace. En effet, l'expérience contrôle mesurant l'internalisation d'un mutant *fnbAB* confirme l'implication des FnBPs dans l'invasion de ces cellules par *S. aureus* (Figure 7B). Les Figures 7A et 7C montrent que SigB est nécessaire à la persistance des SCVs dans les cellules FK.

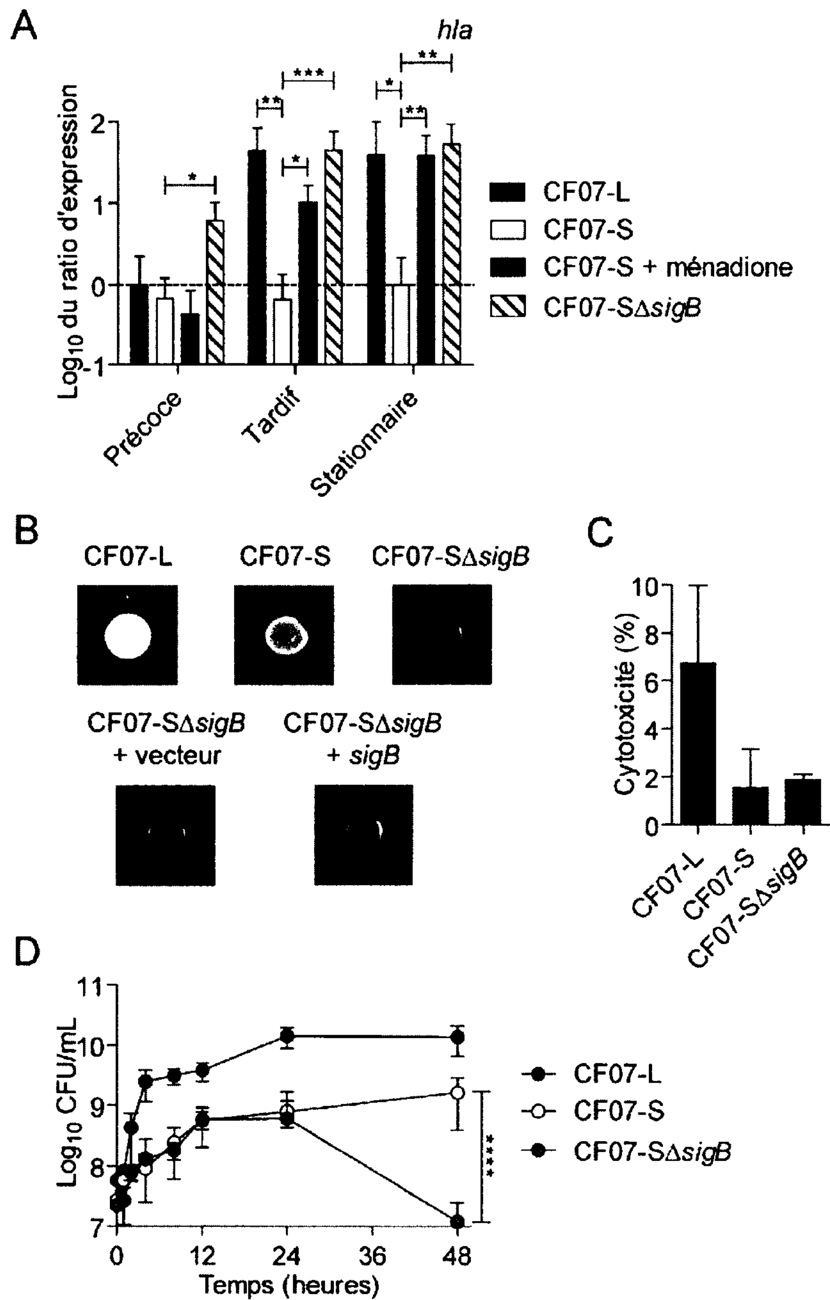


FIGURE 8. SigB module l'activité hémolytique et la survie en phase stationnaire des SCVs. (A) Cinétiques de ratio d'expression d'hla en fonction de la croissance tel qu'évaluées par PCR quantitative en temps réel. Les différences significatives par rapport à CF07-S sont indiquées (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) et ont été déterminées par l'ANOVA à

un facteur suivi d'un post-test de Dunnett (n=3-5). Les données sont exprimées par rapport à CF07-L en phase de croissance exponentielle précoce. (B) Évaluation de l'activité hémolytique de CF07-L, CF07-S, CF07-SΔ*sigB*, CF07-SΔ*sigB* + vecteur vide et CF07-SΔ*sigB* + vecteur *sigB* incubés pendant 48 heures sur pétris-sang en présence de $CdCl_2$ (0.25 μM). (C) Évaluation de la lyse cellulaire durant des infections de 48 heures de cellules épithéliales pulmonaires FK avec CF07-L, CF07-S et CF07-SΔ*sigB* à l'aide d'essais de cytotoxicité à la lactate déshydrogénase. Un ANOVA à un facteur suivi d'un post-test de Tuckey ne révèle aucune différence significative (n=2). (D) Courbes de croissance de CF07-L, CF07-S et CF07-SΔ*sigB*. La différence significative entre CF07-S et CF07-SΔ*sigB* est indiquée (****, $P < 0.0001$) et a été déterminée par l'ANOVA à deux facteurs suivi d'un post-test de Bonferonni (n=3-6). Les données sont représentées par des moyennes avec les déviations standards.

INTERPRÉTATION DES RÉSULTATS : Certaines études suggèrent que SigB influence l'induction de la mort de la cellule hôte lors d'infections à *S. aureus* (Haslinger-Loffler *et al.*, 2005; Kubica *et al.*, 2008). L'α-hémolysine (Hla) pourrait être un facteur important à cette induction (Jarry *et al.*, 2008; Kubica *et al.*, 2008; Sinha et Fraunholz, 2010) et est sous le contrôle de SigB (Horsburgh *et al.*, 2002). La Figure 8A montre que *hla* est très faiblement exprimé chez CF07-S en comparaison à CF07-L et CF07-S en présence de ménadione. De plus, l'expression d'HLA est fortement induite chez CF07-SΔ*sigB* en comparaison à CF07-S. Les résultats d'expression d'HLA corroborent ceux d'hémolyse montrant que SigB est responsable de l'inhibition de l'activité hémolytique chez CF07-S. Cependant, la cytotoxicité induite par CF07-S et CF07-SΔ*sigB* n'est pas différente lors d'infections de cellules épithéliales pulmonaires FK (Figure 8C). Ces résultats suggèrent que le rôle de SigB dans la persistance intracellulaire des SCVs n'implique pas le contrôle de l'induction de la mort cellulaire, mais plutôt la réplication ou la survie intracellulaire. Il est connu que SigB joue un rôle dans la résistance à plusieurs stress tels que ceux rencontrés en phase de croissance stationnaire (Figure 8D). Certains de ces stress pourraient être rencontrés par les SCVs au cours du cycle d'infection intracellulaire (voir Discussion).

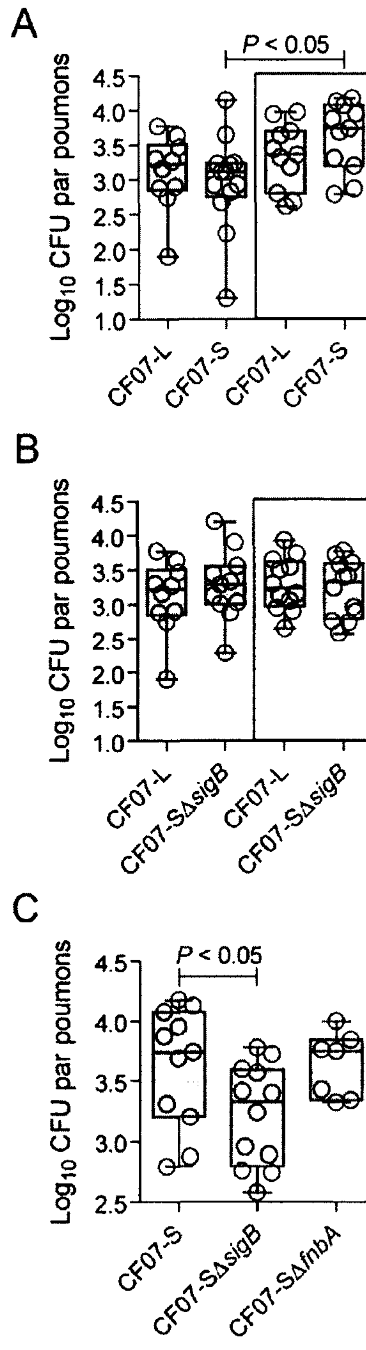


FIGURE 9. Rôle du phénotype SCV, de SigB et de FnBPA lors d'infections simples et combinants des souches normales et SCVs dans un modèle d'infection pulmonaire murin. (A)

CFU récupérés de poumons de souris infectés avec CF07-L, CF07-S et une combinaison des deux souches 48 heures post-inoculation. Une différence significative est indiquée (*, $P < 0.05$) et a été déterminée par un test de Kruskal-Wallis suivi d'un post-test de Dunn. (B) CFU récupérés de poumons de souris infectés avec CF07-L, CF07-SΔ*sigB* et une combinaison des deux souches 48 heures post-inoculation. Un test de Kruskal-Wallis suivi d'un post-test de Dunn ne révèle aucune différence significative. (C) CFU récupérés de poumons de souris infectés avec CF07-S, CF07-SΔ*sigB* et CF07-SΔ*fnbA* en présence de CF07-L 48 heures post-inoculation. Une différence significative est indiquée (*, $P < 0.05$) et a été déterminée par un test de Kruskal-Wallis suivi d'un post-test de Dunn.

INTERPRÉTATION DES RÉSULTATS : Comme discuté précédemment, le rôle des SCVs lors d'infections à *S. aureus* n'a pas été démontré clairement dans les modèles d'infections expérimentaux. Pratiquement aucune information n'est disponible concernant la contribution *in vivo* de régulateurs ou facteurs de virulence à la pathogenèse des SCVs. Il est important de noter que les SCVs sont habituellement retrouvés simultanément avec des bactéries au phénotype normal chez les patients FK (Proctor *et al.*, 2006). Les Figures 9A-C montrent que les SCVs sont favorisés lors d'infections pulmonaires combinées avec des souches normales et que SigB (mais pas FnBPA) est important à ce phénomène.

BIBLIOGRAPHIE

Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C., and Tarkowski, A. (1993). The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. Infect. Immun. *61*, 3879-3885.

Abu-Qatouseh, L.F., Chinni, S.V., Seggewiss, J., Proctor, R.A., Brosius, J., Rozhdestvensky, T.S., Peters, G., von Eiff, C., and Becker, K. (2010). Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. J. Mol. Med. (Berl) *88*, 565-575.

Acar, J.F., Goldstein, F.W., and Lagrange, P. (1978). Human infections caused by thiamine- or menadione-requiring *Staphylococcus aureus*. J. Clin. Microbiol. *8*, 142-147.

Aizenman, E., Engelberg-Kulka, H., and Glaser, G. (1996). An *Escherichia coli* chromosomal "addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: a model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA *93*, 6059-6063.

Al Laham, N., Rohde, H., Sander, G., Fischer, A., Hussain, M., Heilmann, C., Mack, D., Proctor, R., Peters, G., Becker, K., *et al.* (2007). Augmented expression of polysaccharide intercellular adhesin in a defined *Staphylococcus epidermidis* mutant with the small-colony-variant phenotype. J. Bacteriol. *189*, 4494-4501.

Albus, A., Fournier, J.M., Wolz, C., Boutonnier, A., Ranke, M., Hoiby, N., Hochkeppel, H., and Doring, G. (1988). *Staphylococcus aureus* capsular types and antibody response to lung infection in patients with cystic fibrosis. J. Clin. Microbiol. *26*, 2505-2509.

Aldallal, N., McNaughton, E.E., Manzel, L.J., Richards, A.M., Zabner, J., Ferkol, T.W., and Look, D.C. (2002). Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. Am. J. Respir. Crit. Care Med. *166*, 1248-1256.

Archer, G.L. (1998). *Staphylococcus aureus*: a well-armed pathogen. Clin. Infect. Dis. *26*, 1179-1181.

Archer, N.K., Mazaitis, M.J., Costerton, J.W., Leid, J.G., Powers, M.E., and Shirtliff, M.E. (2011). *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. Virulence *2*, 445-459.

Armstrong, D.S., Grimwood, K., Carlin, J.B., Carzino, R., Gutierrez, J.P., Hull, J., Olinsky, A., Phelan, E.M., Robertson, C.F., and Phelan, P.D. (1997). Lower airway inflammation in

infants and young children with cystic fibrosis. Am. J. Respir. Crit. Care Med. *156*, 1197-1204.

Armstrong, D.S., Hook, S.M., Jamsen, K.M., Nixon, G.M., Carzino, R., Carlin, J.B., Robertson, C.F., and Grimwood, K. (2005). Lower airway inflammation in infants with cystic fibrosis detected by newborn screening. Pediatr. Pulmonol. *40*, 500-510.

Atalla, H., Gyles, C., Jacob, C.L., Moisan, H., Malouin, F., and Mallard, B. (2008). Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. Foodborne Pathog. Dis. *5*, 785-799.

Bakkal, S., Robinson, S.M., Ordonez, C.L., Waltz, D.A., and Riley, M.A. (2010). Role of bacteriocins in mediating interactions of bacterial isolates taken from cystic fibrosis patients. Microbiology *156*, 2058-2067.

Baltch, A.L., Ritz, W.J., Bopp, L.H., Michelsen, P., and Smith, R.P. (2008). Activities of daptomycin and comparative antimicrobials, singly and in combination, against extracellular and intracellular *Staphylococcus aureus* and its stable small-colony variant in human monocyte-derived macrophages and in broth. Antimicrob. Agents Chemother. *52*, 1829-1833.

Balwit, J.M., van Langevelde, P., Vann, J.M., and Proctor, R.A. (1994). Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J. Infect. Dis. *170*, 1033-1037.

Baran, J., Weglarczyk, K., Mysiak, M., Guzik, K., Ernst, M., Flad, H.D., and Pryjma, J. (2001). Fas (CD95)-Fas ligand interactions are responsible for monocyte apoptosis occurring as a result of phagocytosis and killing of *Staphylococcus aureus*. Infect. Immun. *69*, 1287-1297.

Bates, D.M., von Eiff, C., McNamara, P.J., Peters, G., Yeaman, M.R., Bayer, A.S., and Proctor, R.A. (2003). *Staphylococcus aureus menD* and *hemB* mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. J. Infect. Dis. *187*, 1654-1661.

Batoni, G., Maisetta, G., Brancatisano, F.L., Esin, S., and Campa, M. (2011). Use of antimicrobial peptides against microbial biofilms: advantages and limits. Curr. Med. Chem. *18*, 256-279.

Baumert, N., von Eiff, C., Schaaff, F., Peters, G., Proctor, R.A., and Sahl, H.G. (2002). Physiology and antibiotic susceptibility of *Staphylococcus aureus* small colony variants. Microb. Drug Resist. *8*, 253-260.

Bayles, K.W., Wesson, C.A., Liou, L.E., Fox, L.K., Bohach, G.A., and Trumble, W.R. (1998). Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. Infect. Immun. *66*, 336-342.

Bednarek, P., and Osbourn, A. (2009). Plant-microbe interactions: chemical diversity in plant defense. Science *324*, 746-748.

Beenken, K.E., Blevins, J.S., and Smeltzer, M.S. (2003). Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. Infect. Immun. *71*, 4206-4211.

Beenken, K.E., Mrak, L.N., Griffin, L.M., Zielinska, A.K., Shaw, L.N., Rice, K.C., Horswill, A.R., Bayles, K.W., and Smeltzer, M.S. (2010). Epistatic relationships between *sarA* and *agr* in *Staphylococcus aureus* biofilm formation. PLoS One *5*, e10790.

Beveridge, T.J., Makin, S.A., Kadurugamuwa, J.L., and Li, Z. (1997). Interactions between biofilms and the environment. FEMS Microbiol. Rev. *20*, 291-303.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B., and Projan, S. (2004). Microarray-based analysis of the *Staphylococcus aureus* sigmaB regulon. J. Bacteriol. *186*, 4085-4099.

Bischoff, M., Entenza, J.M., and Giachino, P. (2001). Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. J. Bacteriol. *183*, 5171-5179.

Biswas, L., Biswas, R., Schlag, M., Bertram, R., and Gotz, F. (2009). Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. *75*, 6910-6912.

Bjarnsholt, T., Jensen, P.O., Rasmussen, T.B., Christophersen, L., Calum, H., Hentzer, M., Hougen, H.P., Rygaard, J., Moser, C., Eberl, L., *et al.* (2005). Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. Microbiology *151*, 3873-3880.

Black, H.R., Yankaskas, J.R., Johnson, L.G., and Noah, T.L. (1998). Interleukin-8 production by cystic fibrosis nasal epithelial cells after tumor necrosis factor-alpha and respiratory syncytial virus stimulation. Am. J. Respir. Cell Mol. Biol. *19*, 210-215.

Boles, B.R., and Horswill, A.R. (2008). *agr*-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog. *4*, e1000052.

Bonfield, T.L., Konstan, M.W., and Berger, M. (1999). Altered respiratory epithelial cell cytokine production in cystic fibrosis. J. Allergy Clin. Immunol. *104*, 72-78.

Bouarab, K., Melton, R., Peart, J., Baulcombe, D., and Osbourn, A. (2002). A saponin-detoxifying enzyme mediates suppression of plant defences. Nature *418*, 889-892.

Boyd, A., and Chakrabarty, A.M. (1994). Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. *60*, 2355-2359.

Brady, R.A., Leid, J.G., Camper, A.K., Costerton, J.W., and Shirtliff, M.E. (2006). Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. Infect. Immun. *74*, 3415-3426.

Bronner, S., Monteil, H., and Prevost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiol. Rev. *28*, 183-200.

Brouillette, E., Grondin, G., Shkreta, L., Lacasse, P., and Talbot, B.G. (2003a). *In vivo* and *in vitro* demonstration that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. Microb. Pathog. *35*, 159-168.

Brouillette, E., Martinez, A., Boyll, B.J., Allen, N.E., and Malouin, F. (2004). Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*. FEMS Immunol. Med. Microbiol. *41*, 35-41.

Brouillette, E., Talbot, B.G., and Malouin, F. (2003b). The fibronectin-binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis. Infect. Immun. *71*, 2292-2295.

Bulger, R.J., and Bulger, R.E. (1967). Ultrastructure of small colony variants of a methicillin-resistant Staphylococcus aureus. J. Bacteriol. *94*, 1244-1246.

Canadian Cystic Fibrosis Foundation (2009). Patient data registry report. Toronton, ON, Canada.

Cassat, J., Dunman, P.M., Murphy, E., Projan, S.J., Beenken, K.E., Palm, K.J., Yang, S.J., Rice, K.C., Bayles, K.W., and Smeltzer, M.S. (2006). Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology *152*, 3075-3090.

Cebrian, G., Sagarzazu, N., Aertsen, A., Pagan, R., Condon, S., and Manas, P. (2009). Role of the alternative sigma factor sigmaB on *Staphylococcus aureus* resistance to stresses of relevance to food preservation. J. Appl. Microbiol. *107*, 187-196.

Chambers, H.F., and Deleo, F.R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. *7*, 629-641.

Chan, P.F., Foster, S.J., Ingham, E., and Clements, M.O. (1998). The *Staphylococcus aureus* alternative sigma factor sigmaB controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J. Bacteriol. *180*, 6082-6089.

Chan, W.C., Coyle, B.J., and Williams, P. (2004). Virulence regulation and quorum sensing in staphylococcal infections: competitive AgrC antagonists as quorum sensing inhibitors. J. Med. Chem. *47*, 4633-4641.

Chatterjee, I., Kriegeskorte, A., Fischer, A., Deiwick, S., Theimann, N., Proctor, R.A., Peters, G., Herrmann, M., and Kahl, B.C. (2008). *In vivo* mutations of thymidylate synthase (encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. J. Bacteriol. *190*, 834-842.

Chattoraj, S.S., Murthy, R., Ganesan, S., Goldberg, J.B., Zhao, Y., Hershenson, M.B., and Sajjan, U.S. (2010). *Pseudomonas aeruginosa* alginate promotes *Burkholderia cenocepacia* persistence in cystic fibrosis transmembrane conductance regulator knockout mice. Infect. Immun. *78*, 984-993.

Chen, W., Kuolee, R., and Yan, H. (2010). The potential of 3',5'-cyclic diguanylic acid (c-di-GMP) as an effective vaccine adjuvant. Vaccine *28*, 3080-3085.

Cheung, A.L., Eberhardt, K.J., Chung, E., Yeaman, M.R., Sullam, P.M., Ramos, M., and Bayer, A.S. (1994). Diminished virulence of a *sar-/agr-* mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. J. Clin. Invest. *94*, 1815-1822.

Cheung, A.L., Schmidt, K., Bateman, B., and Manna, A.C. (2001). SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. Infect. Immun. *69*, 2448-2455.

Cheung, A.L., Yang, S.J., Bayer, A.S., and Xiong, Y.Q. (2009). Disparity in the *in vitro* versus *in vivo* regulation of fibronectin-binding proteins by 2 global regulators, *saeRS* and *sigB*, in *Staphylococcus aureus*. J. Infect. Dis. *200*, 1371-1374.

Cheung, A.L., and Zhang, G. (2002). Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. Front Biosci. *7*, d1825-1842.

Chmiel, J.F., and Davis, P.B. (2003). State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? Respir. Res. *4*, 8.

Clements, M.O., Watson, S.P., Poole, R.K., and Foster, S.J. (1999). CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis. J. Bacteriol. *181*, 501-507.

Cole, A.M., and Ganz, T. (2002). Antimicrobial peptides and proteins in the CF airway. Methods Mol. Med. *70*, 447-464.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. Science *284*, 1318-1322.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., and Gotz, F. (1999). The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. *67*, 5427-5433.

Cramton, S.E., Ulrich, M., Gotz, F., and Doring, G. (2001). Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Infect. Immun. *69*, 4079-4085.

Cuerrier, C.M., Benoit, M., Guillemette, G., Gobeil, F., Jr., and Grandbois, M. (2009). Real-time monitoring of angiotensin II-induced contractile response and cytoskeleton remodeling in individual cells by atomic force microscopy. Pflugers Arch. *457*, 1361-1372.

Cui, L., Neoh, H.M., Iwamoto, A., and Hiramatsu, K. (2012). Coordinated phenotype switching with large-scale chromosome flip-flop inversion observed in bacteria. Proc. Natl. Acad. Sci. USA *109*, E1647-1656.

Cystic Fibrosis Foundation (2009). Patient registry annual report, Washington, D.C.

Dales, L., Ferris, W., Vandemheen, K., and Aaron, S.D. (2009). Combination antibiotic susceptibility of biofilm-grown *Burkholderia cepacia* and *Pseudomonas aeruginosa* isolated from patients with pulmonary exacerbations of cystic fibrosis. Eur. J. Clin. Microbiol. Infect. Dis. *28*, 1275-1279.

Dasenbrook, E.C., Checkley, W., Merlo, C.A., Konstan, M.W., Lechtzin, N., and Boyle, M.P. (2010). Association between respiratory tract methicillin-resistant *Staphylococcus aureus* and survival in cystic fibrosis. JAMA *303*, 2386-2392.

Dasenbrook, E.C., Merlo, C.A., Diener-West, M., Lechtzin, N., and Boyle, M.P. (2008). Persistent methicillin-resistant *Staphylococcus aureus* and rate of FEV1 decline in cystic fibrosis. Am. J. Respir. Crit. Care Med. *178*, 814-821.

Davies, J.C., and Bilton, D. (2009). Bugs, biofilms, and resistance in cystic fibrosis. Respir. Care *54*, 628-640.

Davis, P.B., Drumm, M., and Konstan, M.W. (1996). Cystic fibrosis. Am. J. Respir. Crit. Care Med. *154*, 1229-1256.

De Beer, D., Srinivasan, R., and Stewart, P.S. (1994). Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. *60*, 4339-4344.

Deitch, E.A., Haskel, Y., Cruz, N., Xu, D., and Kvietys, P.R. (1995). Caco-2 and IEC-18 intestinal epithelial cells exert bactericidal activity through an oxidant-dependent pathway. Shock *4*, 345-350.

Deora, R., Tseng, T., and Misra, T.K. (1997). Alternative transcription factor sigmaB of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *sar*. J. Bacteriol. *179*, 6355-6359.

Depke, M., Burian, M., Schafer, T., Broker, B.M., Ohlsen, K., and Volker, U. (2012). The alternative sigma factor B modulates virulence gene expression in a murine *Staphylococcus aureus* infection model but does not influence kidney gene expression pattern of the host. Int. J. Med. Microbiol. *302*, 33-39.

Donegan, N.P., and Cheung, A.L. (2009). Regulation of the *mazEF* toxin-antitoxin module in *Staphylococcus aureus* and its impact on *sigB* expression. J. Bacteriol. *191*, 2795-2805.

Donegan, N.P., Thompson, E.T., Fu, Z., and Cheung, A.L. (2010). Proteolytic regulation of toxin-antitoxin systems by ClpPC in *Staphylococcus aureus*. J. Bacteriol. *192*, 1416-1422.

Donlan, R.M., and Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. *15*, 167-193.

Duan, K., Dammel, C., Stein, J., Rabin, H., and Surette, M.G. (2003). Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol. Microbiol. *50*, 1477-1491.

Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E.L., Zagursky, R.J., Shlaes, D., and Projan, S.J. (2001). Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. *183*, 7341-7353.

Ebensen, T., Libanova, R., Schulze, K., Yevsa, T., Morr, M., and Guzman, C.A. (2010). Bis-(3',5')-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. Vaccine *29*, 5210-5220.

Elizur, A., Cannon, C.L., and Ferkol, T.W. (2008). Airway inflammation in cystic fibrosis. Chest *133*, 489-495.

Engelhardt, J.F., Yang, Y., Stratford-Perricaudet, L.D., Allen, E.D., Kozarsky, K., Perricaudet, M., Yankaskas, J.R., and Wilson, J.M. (1993). Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. Nat. Genet. *4*, 27-34.

Entenza, J.M., Moreillon, P., Senn, M.M., Kormanec, J., Dunman, P.M., Berger-Bachi, B., Projan, S., and Bischoff, M. (2005). Role of sigmaB in the expression of *Staphylococcus aureus* cell wall adhesins ClfA and FnbA and contribution to infectivity in a rat model of experimental endocarditis. Infect. Immun. *73*, 990-998.

European Cystic Fibrosis Society (2009). Patient Registry Report.

Fitzpatrick, F., Humphreys, H., and O'Gara, J.P. (2005). The genetics of staphylococcal biofilm formation--will a greater understanding of pathogenesis lead to better management of device-related infection? Clin. Microbiol. Infect. *11*, 967-973.

Foster, T.J. (2005). Immune evasion by staphylococci. Nat. Rev. Microbiol. *3*, 948-958.

Foster, T.J. (2009). Colonization and infection of the human host by staphylococci: adhesion, survival and immune evasion. Vet. Dermatol. *20*, 456-470.

Fournier, B., and Hooper, D.C. (2000). A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J. Bacteriol. *182*, 3955-3964.

Foweraker, J.E., Laughton, C.R., Brown, D.F., and Bilton, D. (2005). Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. J. Antimicrob. Chemother. *55*, 921-927.

Friedman, M. (2002). Tomato glycoalkaloids: role in the plant and in the diet. J. Agric. Food Chem. *50*, 5751-5780.

Fu, Z., Donegan, N.P., Memmi, G., and Cheung, A.L. (2007). Characterization of MazFSa, an endoribonuclease from *Staphylococcus aureus*. J. Bacteriol. *189*, 8871-8879.

Fu, Z., Tamber, S., Memmi, G., Donegan, N.P., and Cheung, A.L. (2009). Overexpression of MazFsa in *Staphylococcus aureus* induces bacteriostasis by selectively targeting mRNAs for cleavage. J. Bacteriol. *191*, 2051-2059.

Gadsby, D.C., and Nairn, A.C. (1999). Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. Physiol. Rev. *79*, S77-S107.

Galli, J., Ardito, F., Calo, L., Mancinelli, L., Imperiali, M., Parrilla, C., Picciotti, P.M., and Fadda, G. (2007). Recurrent upper airway infections and bacterial biofilms. J. Laryngol. Otol. *121*, 341-344.

Garzoni, C., and Kelley, W.L. (2009). *Staphylococcus aureus*: new evidence for intracellular persistence. Trends Microbiol. *17*, 59-65.

George, A.M., Jones, P.M., and Middleton, P.G. (2009). Cystic fibrosis infections: treatment strategies and prospects. FEMS Microbiol. Lett. *300*, 153-164.

Gerdes, K., Christensen, S.K., and Lobner-Olesen, A. (2005). Prokaryotic toxin-antitoxin stress response loci. Nat. Rev. Microbiol. *3*, 371-382.

Gertz, S., Engelmann, S., Schmid, R., Ohlsen, K., Hacker, J., and Hecker, M. (1999). Regulation of sigmaB-dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus* strains. Mol. Gen. Genet. *261*, 558-566.

Giachino, P., Engelmann, S., and Bischoff, M. (2001). Sigma(B) activity depends on RsbU in *Staphylococcus aureus*. J. Bacteriol. *183*, 1843-1852.

Gibson, R.L., Burns, J.L., and Ramsey, B.W. (2003). Pathophysiology and management of pulmonary infections in cystic fibrosis. Am. J. Respir. Crit. Care Med. *168*, 918-951.

Giese, B., Dittmann, S., Paprotka, K., Levin, K., Weltrowski, A., Biehler, D., Lam, T.T., Sinha, B., and Fraunholz, M.J. (2009). Staphylococcal alpha-toxin is not sufficient to mediate escape from phagolysosomes in upper-airway epithelial cells. Infect. Immun. *77*, 3611-3625.

Giese, B., Glowinski, F., Paprotka, K., Dittmann, S., Steiner, T., Sinha, B., and Fraunholz, M.J. (2011). Expression of delta-toxin by *Staphylococcus aureus* mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of beta-toxin. Cell Microbiol. *13*, 316-329.

Gillaspy, A.F., Hickmon, S.G., Skinner, R.A., Thomas, J.R., Nelson, C.L., and Smeltzer, M.S. (1995). Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. Infect. Immun. *63*, 3373-3380.

Gilligan, P.H., Gage, P.A., Welch, D.F., Muszynski, M.J., and Wait, K.R. (1987). Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. J. Clin. Microbiol. *25*, 1258-1261.

Giraudo, A.T., Calzolari, A., Cataldi, A.A., Bogni, C., and Nagel, R. (1999). The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. FEMS Microbiol. Lett. *177*, 15-22.

Giraudo, A.T., Raspanti, C.G., Calzolari, A., and Nagel, R. (1994). Characterization of a Tn551-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. Can. J. Microbiol. *40*, 677-681.

Goerke, C., and Wolz, C. (2004). Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. Int. J. Med. Microbiol. *294*, 195-202.

Goerke, C., and Wolz, C. (2010). Adaptation of *Staphylococcus aureus* to the cystic fibrosis lung. Int. J. Med. Microbiol. *300*, 520-525.

Goss, C.H., and Muhlebach, M.S. (2011). Review: *Staphylococcus aureus* and MRSA in cystic fibrosis. J. Cyst. Fibros. *10*, 298-306.

Gresham, H.D., Lowrance, J.H., Caver, T.E., Wilson, B.S., Cheung, A.L., and Lindberg, F.P. (2000). Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. J. Immunol. *164*, 3713-3722.

Gruber, T.M., and Gross, C.A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. *57*, 441-466.

Grundmeier, M., Tuchscherr, L., Bruck, M., Viemann, D., Roth, J., Willscher, E., Becker, K., Peters, G., and Loffler, B. (2010). Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells. J. Infect. Dis. *201*, 871-880.

Guggino, W.B. (1999). Cystic fibrosis and the salt controversy. Cell *96*, 607-610.

Hanrahan, J.W., and Wioland, M.A. (2004). Revisiting cystic fibrosis transmembrane conductance regulator structure and function. Proc. Am. Thorac. Soc. *1*, 17-21.

Harrison, F. (2007). Microbial ecology of the cystic fibrosis lung. Microbiology *153*, 917-923.

Haslinger-Loffler, B., Kahl, B.C., Grundmeier, M., Strangfeld, K., Wagner, B., Fischer, U., Cheung, A.L., Peters, G., Schulze-Osthoff, K., and Sinha, B. (2005). Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells. Cell Microbiol. *7*, 1087-1097.

Haslinger, B., Strangfeld, K., Peters, G., Schulze-Osthoff, K., and Sinha, B. (2003). *Staphylococcus aureus* alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. Cell Microbiol. *5*, 729-741.

Hauck, C.R., and Ohlsen, K. (2006). Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. Curr. Opin. Microbiol. *9*, 5-11.

Haussler, S. (2004). Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. Environ. Microbiol. *6*, 546-551.

Haussler, S., Lehmann, C., Breselge, C., Rohde, M., Classen, M., Tummler, B., Vandamme, P., and Steinmetz, I. (2003). Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia* complex. Eur. J. Clin. Microbiol. Infect. Dis. *22*, 249-253.

Haussler, S., Tummler, B., Weissbrodt, H., Rohde, M., and Steinmetz, I. (1999). Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. Clin. Infect. Dis. *29*, 621-625.

Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.A., Song, Z., Kristoffersen, P., *et al.* (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. Embo J. *22*, 3803-3815.

Herbert, S., Barry, P., and Novick, R.P. (2001). Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. Infect. Immun. *69*, 2996-3003.

Herbert, S., Ziebandt, A.K., Ohlsen, K., Schafer, T., Hecker, M., Albrecht, D., Novick, R., and Gotz, F. (2010). Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. Infect. Immun. *78*, 2877-2889.

Hoffman, L.R., Deziel, E., D'Argenio, D.A., Lepine, F., Emerson, J., McNamara, S., Gibson, R.L., Ramsey, B.W., and Miller, S.I. (2006). Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA *103*, 19890-19895.

Hogardt, M., and Heesemann, J. (2010). Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. Int. J. Med. Microbiol. *300*, 557-562.

Hoiby, N. (2011). Recent advances in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. BMC Med. *9*, 32.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. Int. J. Antimicrob. Agents *35*, 322-332.

Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K., and Foster, S.J. (2002). sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. J. Bacteriol. *184*, 5457-5467.

Houston, P., Rowe, S.E., Pozzi, C., Waters, E.M., and O'Gara, J.P. (2011). Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. Infect. Immun. *79*, 1153-1165.

Hsieh, H.Y., Tseng, C.W., and Stewart, G.C. (2008). Regulation of Rot expression in *Staphylococcus aureus*. J. Bacteriol. *190*, 546-554.

Hu, Y., Shamaei-Tousi, A., Liu, Y., and Coates, A. (2010). A new approach for the discovery of antibiotics by targeting non-multiplying bacteria: a novel topical antibiotic for staphylococcal infections. PLoS One *5*, e11818.

Hudson, V.L., Wielinski, C.L., and Regelmann, W.E. (1993). Prognostic implications of initial oropharyngeal bacterial flora in patients with cystic fibrosis diagnosed before the age of two years. J. Pediatr. *122*, 854-860.

Hummler, E., Barker, P., Gatzy, J., Beermann, F., Verdumo, C., Schmidt, A., Boucher, R., and Rossier, B.C. (1996). Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. Nat. Genet. *12*, 325-328.

Hunter, M.J., Treharne, K.J., Winter, A.K., Cassidy, D.M., Land, S., and Mehta, A. (2010). Expression of wild-type CFTR suppresses NF-kappaB-driven inflammatory signalling. PLoS One *5*, e11598.

Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E., and Higgins, C.F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature *346*, 362-365.

Inose, Y., Takeshita, S.L., Hidaka, T., Higashide, M., Maruyama, A., Hayashi, H., Morikawa, K., and Ohta, T. (2006). Genetic characterization of the natural SigB variants found in clinical isolates of *Staphylococcus aureus*. J. Gen. Appl. Microbiol. *52*, 259-271.

Jarry, T.M., and Cheung, A.L. (2006). *Staphylococcus aureus* escapes more efficiently from the phagosome of a cystic fibrosis bronchial epithelial cell line than from its normal counterpart. Infect. Immun. *74*, 2568-2577.

Jarry, T.M., Memmi, G., and Cheung, A.L. (2008). The expression of alpha-haemolysin is required for *Staphylococcus aureus* phagosomal escape after internalization in CFT-1 cells. Cell Microbiol. *10*, 1801-1814.

Jayaraman, S., Joo, N.S., Reitz, B., Wine, J.J., and Verkman, A.S. (2001). Submucosal gland secretions in airways from cystic fibrosis patients have normal [Na(+)] and pH but elevated viscosity. Proc. Natl. Acad. Sci. USA *98*, 8119-8123.

Jefferson, K.K., Cramton, S.E., Gotz, F., and Pier, G.B. (2003). Identification of a 5-nucleotide sequence that controls expression of the ica locus in *Staphylococcus aureus* and characterization of the DNA-binding properties of IcaR. Mol. Microbiol. *48*, 889-899.

Jiang, Q., Li, J., Dubroff, R., Ahn, Y.J., Foskett, J.K., Engelhardt, J., and Kleyman, T.R. (2000). Epithelial sodium channels regulate cystic fibrosis transmembrane conductance regulator chloride channels in Xenopus oocytes. J. Biol. Chem. *275*, 13266-13274.

Jonsson, I.M., Arvidson, S., Foster, S., and Tarkowski, A. (2004). Sigma factor B and RsbU are required for virulence in *Staphylococcus aureus*-induced arthritis and sepsis. Infect. Immun. *72*, 6106-6111.

Jonsson, I.M., von Eiff, C., Proctor, R.A., Peters, G., Ryden, C., and Tarkowski, A. (2003). Virulence of a *hemB* mutant displaying the phenotype of a *Staphylococcus aureus* small colony variant in a murine model of septic arthritis. Microb. Pathog. *34*, 73-79.

Joseph, T., Look, D., and Ferkol, T. (2005). NF-kappaB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with *Pseudomonas aeruginosa*. Am. J. Physiol. Lung Cell. Mol. Physiol. *288*, L471-479.

Kahl, B., Herrmann, M., Everding, A.S., Koch, H.G., Becker, K., Harms, E., Proctor, R.A., and Peters, G. (1998). Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. J. Infect. Dis. *177*, 1023-1029.

Kahl, B.C. (2010). Impact of *Staphylococcus aureus* on the pathogenesis of chronic cystic fibrosis lung disease. Int. J. Med. Microbiol. *300*, 514-519.

Kahl, B.C., Belling, G., Becker, P., Chatterjee, I., Wardecki, K., Hilgert, K., Cheung, A.L., Peters, G., and Herrmann, M. (2005). Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infect. Immun. *73*, 4119-4126.

Kahl, B.C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R.A., and Peters, G. (2003a). Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. J. Clin. Microbiol. *41*, 410-413.

Kahl, B.C., Duebbers, A., Lubritz, G., Haeberle, J., Koch, H.G., Ritzerfeld, B., Reilly, M., Harms, E., Proctor, R.A., Herrmann, M., *et al.* (2003b). Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. J. Clin. Microbiol. *41*, 4424-4427.

Kahl, B.C., Goulian, M., van Wamel, W., Herrmann, M., Simon, S.M., Kaplan, G., Peters, G., and Cheung, A.L. (2000). *Staphylococcus aureus* RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line. Infect. Immun. *68*, 5385-5392.

Karaolis, D.K., Means, T.K., Yang, D., Takahashi, M., Yoshimura, T., Muraille, E., Philpott, D., Schroeder, J.T., Hyodo, M., Hayakawa, Y., *et al.* (2007). Bacterial c-di-GMP is an immunostimulatory molecule. J. Immunol. *178*, 2171-2181.

Karlsson-Kanth, A., Tegmark-Wisell, K., Arvidson, S., and Oscarsson, J. (2006). Natural human isolates of *Staphylococcus aureus* selected for high production of proteases and alpha-hemolysin are sigmaB deficient. Int. J. Med. Microbiol. *296*, 229-236.

Karlsson, A., Saravia-Otten, P., Tegmark, K., Morfeldt, E., and Arvidson, S. (2001). Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in *Staphylococcus aureus sarA* mutants due to up-regulation of extracellular proteases. Infect. Immun. *69*, 4742-4748.

Kaufmann, G.F., Sartorio, R., Lee, S.H., Rogers, C.J., Meijler, M.M., Moss, J.A., Clapham, B., Brogan, A.P., Dickerson, T.J., and Janda, K.D. (2005). Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones. Proc. Natl. Acad. Sci. USA *102*, 309-314.

Kessler, E., Safrin, M., Olson, J.C., and Ohman, D.E. (1993). Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J. Biol. Chem. *268*, 7503-7508.

Khan, T.Z., Wagener, J.S., Bost, T., Martinez, J., Accurso, F.J., and Riches, D.W. (1995). Early pulmonary inflammation in infants with cystic fibrosis. Am. J. Respir. Crit. Care Med. *151*, 1075-1082.

Kiedrowski, M.R., Kavanaugh, J.S., Malone, C.L., Mootz, J.M., Voyich, J.M., Smeltzer, M.S., Bayles, K.W., and Horswill, A.R. (2011). Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. PLoS One *6*, e26714.

Kintarak, S., Whawell, S.A., Speight, P.M., Packer, S., and Nair, S.P. (2004). Internalization of *Staphylococcus aureus* by human keratinocytes. Infect. Immun. *72*, 5668-5675.

Kipp, F., Kahl, B.C., Becker, K., Baron, E.J., Proctor, R.A., Peters, G., and von Eiff, C. (2005). Evaluation of two chromogenic agar media for recovery and identification of *Staphylococcus aureus* small-colony variants. J. Clin. Microbiol. *43*, 1956-1959.

Kipp, F., Ziebuhr, W., Becker, K., Krimmer, V., Hobeta, N., Peters, G., and Von Eiff, C. (2003). Detection of *Staphylococcus aureus* by 16S rRNA directed *in situ* hybridisation in a patient with a brain abscess caused by small colony variants. J. Neurol. Neurosurg. Psychiatry *74*, 1000-1002.

Kleerebezem, M., Quadri, L.E., Kuipers, O.P., and de Vos, W.M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiol. *24*, 895-904.

Klein, M., Kronke, M., and Krut, O. (2006). Expression of lysostaphin in HeLa cells protects from host cell killing by intracellular *Staphylococcus aureus*. Med. Microbiol. Immunol. *195*, 159-163.

Knowles, M.R., and Boucher, R.C. (2002). Mucus clearance as a primary innate defense mechanism for mammalian airways. J. Clin. Invest. *109*, 571-577.

Kohler, C., von Eiff, C., Peters, G., Proctor, R.A., Hecker, M., and Engelmann, S. (2003). Physiological characterization of a heme-deficient mutant of *Staphylococcus aureus* by a proteomic approach. J. Bacteriol. *185*, 6928-6937.

Kong, K.F., Vuong, C., and Otto, M. (2006). *Staphylococcus* quorum sensing in biofilm formation and infection. Int. J. Med. Microbiol. *296*, 133-139.

Koo, S.P., Bayer, A.S., Sahl, H.G., Proctor, R.A., and Yeaman, M.R. (1996). Staphylocidal action of thrombin-induced platelet microbicidal protein is not solely dependent on transmembrane potential. Infect. Immun. *64*, 1070-1074.

Korgaonkar, A.K., and Whiteley, M. (2011). *Pseudomonas aeruginosa* enhances production of an antimicrobial in response to N-acetylglucosamine and peptidoglycan. J. Bacteriol. *193*, 909-917.

Kristian, S.A., Golda, T., Ferracin, F., Cramton, S.E., Neumeister, B., Peschel, A., Gotz, F., and Landmann, R. (2004). The ability of biofilm formation does not influence virulence of *Staphylococcus aureus* and host response in a mouse tissue cage infection model. Microb. Pathog. *36*, 237-245.

Krut, O., Utermohlen, O., Schlossherr, X., and Kronke, M. (2003). Strain-specific association of cytotoxic activity and virulence of clinical *Staphylococcus aureus* isolates. Infect. Immun. *71*, 2716-2723.

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., Golda, A., Maciag-Gudowska, A., Brix, K., Shaw, L., *et al.* (2008). A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. PLoS One *3*, e1409.

Kukavica-Ibrulj, I., and Levesque, R.C. (2008). Animal models of chronic lung infection with *Pseudomonas aeruginosa*: useful tools for cystic fibrosis studies. Lab. Anim. *42*, 389-412.

Kullik, I.I., and Giachino, P. (1997). The alternative sigma factor sigmaB in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. Arch. Microbiol. *167*, 151-159.

Kusch, K., Hanke, K., Holtfreter, S., Schmudde, M., Kohler, C., Erck, C., Wehland, J., Hecker, M., Ohlsen, K., Broker, B., *et al.* (2011). The influence of SaeRS and sigma(B) on the expression of superantigens in different *Staphylococcus aureus* isolates. Int. J. Med. Microbiol. *301*, 488-499.

Lam, T.T., Giese, B., Chikkaballi, D., Kuhn, A., Wolber, W., Pane-Farre, J., Schafer, D., Engelmann, S., Fraunholz, M., and Sinha, B. (2010). Phagolysosomal integrity is generally maintained after *Staphylococcus aureus* invasion of nonprofessional phagocytes but is modulated by strain 6850. Infect. Immun. *78*, 3392-3403.

Lamontagne, C.A., Cuerrier, C.M., and Grandbois, M. (2008). AFM as a tool to probe and manipulate cellular processes. Pflugers Arch. *456*, 61-70.

Lannergard, J., von Eiff, C., Sander, G., Cordes, T., Seggewiss, J., Peters, G., Proctor, R.A., Becker, K., and Hughes, D. (2008). Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus.* Antimicrob. Agents Chemother. *52*, 4017-4022.

Lasa, I., and Penades, J.R. (2006). Bap: a family of surface proteins involved in biofilm formation. Res. Microbiol. *157*, 99-107.

Lauderdale, K.J., Boles, B.R., Cheung, A.L., and Horswill, A.R. (2009). Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infect. Immun. *77*, 1623-1635.

Lehrer, R.I., and Ganz, T. (1999). Antimicrobial peptides in mammalian and insect host defence. Curr. Opin. Immunol. *11*, 23-27.

Lewis, K. (2007). Persister cells, dormancy and infectious disease. Nat. Rev. Microbiol. *5*, 48-56.

Lewis, K. (2010). Persister cells. Annu. Rev. Microbiol. *64*, 357-372.

Lorenz, U., Huttinger, C., Schafer, T., Ziebuhr, W., Thiede, A., Hacker, J., Engelmann, S., Hecker, M., and Ohlsen, K. (2008). The alternative sigma factor sigma B of *Staphylococcus aureus* modulates virulence in experimental central venous catheter-related infections. Microbes Infect. *10*, 217-223.

Lowy, F.D. (1998). *Staphylococcus aureus* infections. N. Engl. J. Med. *339*, 520-532.

Lyczak, J.B., Cannon, C.L., and Pier, G.B. (2002). Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. *15*, 194-222.

Machen, T.E. (2006). Innate immune response in CF airway epithelia: hyperinflammatory? Am. J. Physiol. Cell.Physiol. *291*, C218-230.

Macia, M.D., Blanquer, D., Togores, B., Sauleda, J., Perez, J.L., and Oliver, A. (2005). Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob. Agents Chemother. *49*, 3382-3386.

Magnuson, R.D. (2007). Hypothetical functions of toxin-antitoxin systems. J. Bacteriol. *189*, 6089-6092.

Mann, E.E., Rice, K.C., Boles, B.R., Endres, J.L., Ranjit, D., Chandramohan, L., Tsang, L.H., Smeltzer, M.S., Horswill, A.R., and Bayles, K.W. (2009). Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One *4*, e5822.

Manna, A., and Cheung, A.L. (2001). Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. Infect. Immun. *69*, 885-896.

Manna, A.C., Bayer, M.G., and Cheung, A.L. (1998). Transcriptional analysis of different promoters in the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. *180*, 3828-3836.

Maor, R., and Shirasu, K. (2005). The arms race continues: battle strategies between plants and fungal pathogens. Curr. Opin. Microbiol. *8*, 399-404.

Marti, M., Trotonda, M.P., Tormo-Mas, M.A., Vergara-Irigaray, M., Cheung, A.L., Lasa, I., and Penades, J.R. (2010). Extracellular proteases inhibit protein-dependent biofilm formation in *Staphylococcus aureus*. Microbes Infect. *12*, 55-64.

Martin-Hernandez, A.M., Dufresne, M., Hugouvieux, V., Melton, R., and Osbourn, A. (2000). Effects of targeted replacement of the tomatinase gene on the interaction of *Septoria lycopersici* with tomato plants. Mol. Plant Microbe Interact. *13*, 1301-1311.

Mashburn, L.M., Jett, A.M., Akins, D.R., and Whiteley, M. (2005). *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture. J. Bacteriol. *187*, 554-566.

Massey, R.C., Buckling, A., and Peacock, S.J. (2001). Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. Curr. Biol. *11*, 1810-1814.

Matsui, H., Grubb, B.R., Tarran, R., Randell, S.H., Gatzy, J.T., Davis, C.W., and Boucher, R.C. (1998). Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. Cell *95*, 1005-1015.

Mayer-Hamblett, N., Aitken, M.L., Accurso, F.J., Kronmal, R.A., Konstan, M.W., Burns, J.L., Sagel, S.D., and Ramsey, B.W. (2007). Association between pulmonary function and sputum biomarkers in cystic fibrosis. Am. J. Respir. Crit. Care Med. *175*, 822-828.

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R.P., and Muir, T.W. (1999). Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc. Natl. Acad. Sci. USA *96*, 1218-1223.

McAlester, G., O'Gara, F., and Morrissey, J.P. (2008). Signal-mediated interactions between *Pseudomonas aeruginosa* and *Candida albicans*. J. Med. Microbiol. *57*, 563-569.

McNamara, P.J., Milligan-Monroe, K.C., Khalili, S., and Proctor, R.A. (2000). Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. *182*, 3197-3203.

McNamara, P.J., and Proctor, R.A. (2000). *Staphylococcus aureus* small colony variants, electron transport and persistent infections. Int. J. Antimicrob. Agents *14*, 117-122.

Meier, S., Goerke, C., Wolz, C., Seidl, K., Homerova, D., Schulthess, B., Kormanec, J., Berger-Bachi, B., and Bischoff, M. (2007). sigmaB and the sigmaB-dependent *arlRS* and *yabJ-spoVG* loci affect capsule formation in *Staphylococcus aureus*. Infect. Immun. *75*, 4562-4571.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., Lopez, J.A., Foster, T.J., Penades, J.R., and Lasa, I. (2009). Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J. Bacteriol. *191*, 832-843.

Mittenhuber, G. (1999). Occurrence of *mazEF*-like antitoxin/toxin systems in bacteria. Journal of molecular microbiology and biotechnology *1*, 295-302.

Mohtar, M., Johari, S.A., Li, A.R., Isa, M.M., Mustafa, S., Ali, A.M., and Basri, D.F. (2009). Inhibitory and resistance-modifying potential of plant-based alkaloids against methicillin-resistant *Staphylococcus aureus* (MRSA). Curr. Microbiol. *59*, 181-186.

Moisan, H., Brouillette, E., Jacob, C.L., Langlois-Begin, P., Michaud, S., and Malouin, F. (2006). Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J. Bacteriol. *188*, 64-76.

Molina, A., Del Campo, R., Maiz, L., Morosini, M.I., Lamas, A., Baquero, F., and Canton, R. (2008). High prevalence in cystic fibrosis patients of multiresistant hospital-acquired methicillin-resistant *Staphylococcus aureus* ST228-SCC*mec*I capable of biofilm formation. J. Antimicrob. Chemother. *62*, 961-967.

Morfeldt, E., Janzon, L., Arvidson, S., and Lofdahl, S. (1988). Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. Mol. Gen. Genet. *211*, 435-440.

Moskowitz, S.M., Gibson, R.L., and Effmann, E.L. (2005). Cystic fibrosis lung disease: genetic influences, microbial interactions, and radiological assessment. Pediatr. Radiol. *35*, 739-757.

Mulcahy, L.R., Burns, J.L., Lory, S., and Lewis, K. (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J. Bacteriol. *192*, 6191-6199.

Nair, S.P., Bischoff, M., Senn, M.M., and Berger-Bachi, B. (2003). The sigma B regulon influences internalization of *Staphylococcus aureus* by osteoblasts. Infect. Immun. *71*, 4167-4170.

Nguyen, H.A., Denis, O., Vergison, A., Theunis, A., Tulkens, P.M., Struelens, M.J., and Van Bambeke, F. (2009a). Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: pharmacodynamic evaluation and comparison with isogenic normal-phenotype and revertant strains. Antimicrob. Agents Chemother. *53*, 1434-1442.

Nguyen, H.A., Denis, O., Vergison, A., Tulkens, P.M., Struelens, M.J., and Van Bambeke, F. (2009b). Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: study of antibiotic combinations. Antimicrob. Agents Chemother. *53*, 1443-1449.

Nicholas, R.O., Li, T., McDevitt, D., Marra, A., Sucoloski, S., Demarsh, P.L., and Gentry, D.R. (1999). Isolation and characterization of a *sigB* deletion mutant of *Staphylococcus aureus*. Infect. Immun. *67*, 3667-3669.

Nixon, G.M., Armstrong, D.S., Carzino, R., Carlin, J.B., Olinsky, A., Robertson, C.F., Grimwood, K., and Wainwright, C. (2002). Early airway infection, inflammation, and lung function in cystic fibrosis. Arch. Dis. Child. *87*, 306-311.

Njoroge, J., and Sperandio, V. (2009). Jamming bacterial communication: new approaches for the treatment of infectious diseases. EMBO Mol. Med. *1*, 201-210.

Novick, R.P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. *48*, 1429-1449.

Novick, R.P., and Geisinger, E. (2008). Quorum sensing in staphylococci. Annu. Rev. Genet. *42*, 541-564.

Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. *12*, 3967-3975.

O'Gara, J.P. (2007). *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol. Lett. *270*, 179-188.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D.A., Loughman, A., Foster, T.J., and O'Gara, J.P. (2008). A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J. Bacteriol. *190*, 3835-3850.

O'Neill, E., Pozzi, C., Houston, P., Smyth, D., Humphreys, H., Robinson, D.A., and O'Gara, J.P. (2007). Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. J. Clin. Microbiol. *45*, 1379-1388.

Ooi, N., Miller, K., Randall, C., Rhys-Williams, W., Love, W., and Chopra, I. (2010). XF-70 and XF-73, novel antibacterial agents active against slow-growing and non-dividing cultures of *Staphylococcus aureus* including biofilms. J. Antimicrob. Chemother. *65*, 72-78.

Osbourn, A.E. (1996). Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack. Plant Cell *8*, 1821-1831.

Ostedgaard, L.S., Baldursson, O., and Welsh, M.J. (2001). Regulation of the cystic fibrosis transmembrane conductance regulator Cl- channel by its R domain. J. Biol. Chem. *276*, 7689-7692.

Otto, M. (2008). Staphylococcal biofilms. Curr. Top. Microbiol. Immunol. *322*, 207-228.

Oviedo-Boyso, J., Barriga-Rivera, J.G., Valdez-Alarcon, J.J., Bravo-Patino, A., Carabez-Trejo, A., Cajero-Juarez, M., and Baizabal-Aguirre, V.M. (2008). Internalization of *Staphylococcus aureus* by bovine endothelial cells is associated with the activity state of NF-kappaB and modulated by the pro-inflammatory cytokines TNF-alpha and IL-1beta. Scand. J. Immunol. *67*, 169-176.

Palmer, K.L., Mashburn, L.M., Singh, P.K., and Whiteley, M. (2005). Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. J. Bacteriol. *187*, 5267-5277.

Palmer, M.E., Chaturongakul, S., Wiedmann, M., and Boor, K.J. (2011). The *Listeria monocytogenes* sigmaB regulon and its virulence-associated functions are inhibited by a small molecule. MBio *2*.

Pane-Farre, J., Jonas, B., Forstner, K., Engelmann, S., and Hecker, M. (2006). The sigmaB regulon in *Staphylococcus aureus* and its regulation. Int. J. Med. Microbiol. *296*, 237-258.

Pane-Farre, J., Jonas, B., Hardwick, S.W., Gronau, K., Lewis, R.J., Hecker, M., and Engelmann, S. (2009). Role of RsbU in controlling SigB activity in *Staphylococcus aureus* following alkaline stress. J. Bacteriol. *191*, 2561-2573.

Parkins, M.D., and Elborn, J.S. (2010). Newer antibacterial agents and their potential role in cystic fibrosis pulmonary exacerbation management. J. Antimicrob. Chemother. *65*, 1853-1861.

Patti, J.M., and Hook, M. (1994). Microbial adhesins recognizing extracellular matrix macromolecules. Curr. Opin. Cell Biol. *6*, 752-758.

Patton, T.G., Rice, K.C., Foster, M.K., and Bayles, K.W. (2005). The *Staphylococcus aureus cidC* gene encodes a pyruvate oxidase that affects acetate metabolism and cell death in stationary phase. Mol. Microbiol. *56*, 1664-1674.

Patton, T.G., Yang, S.J., and Bayles, K.W. (2006). The role of proton motive force in expression of the *Staphylococcus aureus cid* and *lrg* operons. Mol. Microbiol. *59*, 1395-1404.

Peng, H.L., Novick, R.P., Kreiswirth, B., Kornblum, J., and Schlievert, P. (1988). Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. *170*, 4365-4372.

Perkins, N.D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat. Rev. Mol. Cell Biol. *8*, 49-62.

Peschel, A. (2002). How do bacteria resist human antimicrobial peptides? Trends Microbiol. *10*, 179-186.

Price, C.W. (2002). General stress response. In Bacillus Subtilis and Its Closest Relatives, A.L. Sonenshein, J.A. Hoch, and R. Losick, eds. (Washington: ASM Press), pp. 369-384.

Pozzi, C., Waters, E.M., Rudkin, J.K., Schaeffer, C.R., Lohan, A.J., Tong, P., Loftus, B.J., Pier, G.B., Fey, P.D., Massey, R.C., *et al.* (2012). Methicillin Resistance Alters the Biofilm Phenotype and Attenuates Virulence in *Staphylococcus aureus* Device-Associated Infections. PLoS Pathog. *8*, e1002626.

Proctor, R.A., van Langevelde, P., Kristjansson, M., Maslow, J.N., and Arbeit, R.D. (1995). Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin. Infect. Dis. *20*, 95-102.

Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M., and Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat. Rev. Microbiol. *4*, 295-305.

Proctor, R.A., and von Humboldt, A. (1998). Bacterial energetics and antimicrobial resistance. Drug. Resist. Updat. *1*, 227-235.

Qazi, S., Middleton, B., Muharram, S.H., Cockayne, A., Hill, P., O'Shea, P., Chhabra, S.R., Camara, M., and Williams, P. (2006). N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in *Staphylococcus aureus*. Infect. Immun. *74*, 910-919.

Qazi, S.N., Counil, E., Morrissey, J., Rees, C.E., Cockayne, A., Winzer, K., Chan, W.C., Williams, P., and Hill, P.J. (2001). *agr* expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. Infect. Immun. *69*, 7074-7082.

Queck, S.Y., Jameson-Lee, M., Villaruz, A.E., Bach, T.H., Khan, B.A., Sturdevant, D.E., Ricklefs, S.M., Li, M., and Otto, M. (2008). RNAIII-independent target gene control by the *agr* quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. Mol. Cell *32*, 150-158.

Rachid, S., Ohlsen, K., Wallner, U., Hacker, J., Hecker, M., and Ziebuhr, W. (2000). Alternative transcription factor sigma(B) is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. J. Bacteriol. *182*, 6824-6826.

Ranganathan, S.C., Parsons, F., Gangell, C., Brennan, S., Stick, S.M., and Sly, P.D. (2011). Evolution of pulmonary inflammation and nutritional status in infants and young children with cystic fibrosis. Thorax *66*, 408-413.

Rani, S.A., Pitts, B., Beyenal, H., Veluchamy, R.A., Lewandowski, Z., Davison, W.M., Buckingham-Meyer, K., and Stewart, P.S. (2007). Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. J. Bacteriol. *189*, 4223-4233.

Rasko, D.A., and Sperandio, V. (2010). Anti-virulence strategies to combat bacteria-mediated disease. Nat. Rev. Drug Discov. *9*, 117-128.

Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Kote, M., Nielsen, J., Eberl, L., and Givskov, M. (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J. Bacteriol. *187*, 1799-1814.

Ratner, A.J., Bryan, R., Weber, A., Nguyen, S., Barnes, D., Pitt, A., Gelber, S., Cheung, A., and Prince, A. (2001). Cystic fibrosis pathogens activate Ca2+-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. J. Biol. Chem. *276*, 19267-19275.

Razvi, S., Quittell, L., Sewall, A., Quinton, H., Marshall, B., and Saiman, L. (2009). Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005. Chest *136*, 1554-1560.

Reddy, K., and Ross, J.M. (2001). Shear stress prevents fibronectin binding protein-mediated *Staphylococcus aureus* adhesion to resting endothelial cells. Infect. Immun. *69*, 3472-3475.

Resch, A., Fehrenbacher, B., Eisele, K., Schaller, M., and Gotz, F. (2005). Phage release from biofilm and planktonic *Staphylococcus aureus* cells. FEMS Microbiol. Lett. *252*, 89-96.

Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., and Bayles, K.W. (2007). The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA *104*, 8113-8118.

Rice, K.C., Patton, T., Yang, S.J., Dumoulin, A., Bischoff, M., and Bayles, K.W. (2004). Transcription of the *Staphylococcus aureus cid* and *lrg* murein hydrolase regulators is affected by sigma factor B. J. Bacteriol. *186*, 3029-3037.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S., and Eberl, L. (2001). N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology *147*, 3249-3262.

Roddick, J.G. (1974). The steroidal glycoalkaloid α-tomatine. Phytochemistry *13*, 9-25.

Rosenfeld, M., Gibson, R.L., McNamara, S., Emerson, J., Burns, J.L., Castile, R., Hiatt, P., McCoy, K., Wilson, C.B., Inglis, A., *et al.* (2001). Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. Pediatr. Pulmonol. *32*, 356-366.

Rothfork, J.M., Dessus-Babus, S., Van Wamel, W.J., Cheung, A.L., and Gresham, H.D. (2003). Fibrinogen depletion attenuates *Staphyloccocus aureus* infection by preventing density-dependent virulence gene up-regulation. J. Immunol. *171*, 5389-5395.

Rubin, B.K. (2007). CFTR is a modulator of airway inflammation. Am. J. Physiol. Lung Cell. Mol. Physiol. *292*, L381-382.

Ruiz-Rubio, M., Perez-Espinosa A., Lairini K., Roldan-Arjona T., Dipietro A., and Anaya N. (2001). Metabolism of the tomato saponin α-tomatine by phytopathogenic fungi. In Studies in Natural Products Chemistry, A. Rahman, ed. (Oxford: Elsevier), pp. 293-326.

Ryan, R.P., and Dow, J.M. (2008). Diffusible signals and interspecies communication in bacteria. Microbiology *154*, 1845-1858.

Ryan, R.P., Fouhy, Y., Garcia, B.F., Watt, S.A., Niehaus, K., Yang, L., Tolker-Nielsen, T., and Dow, J.M. (2008). Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. Mol. Microbiol. *68*, 75-86.

Sadowska, B., Bonar, A., von Eiff, C., Proctor, R.A., Chmiela, M., Rudnicka, W., and Rozalska, B. (2002). Characteristics of *Staphylococcus aureus*, isolated from airways of cystic fibrosis patients, and their small colony variants. FEMS Immunol. Med. Microbiol. *32*, 191-197.

Sagel, S.D., Gibson, R.L., Emerson, J., McNamara, S., Burns, J.L., Wagener, J.S., and Ramsey, B.W. (2009). Impact of *Pseudomonas* and *Staphylococcus* infection on inflammation and clinical status in young children with cystic fibrosis. J. Pediatr. *154*, 183-188.

Saiman, L., Cacalano, G., Gruenert, D., and Prince, A. (1992). Comparison of adherence of *Pseudomonas aeruginosa* to respiratory epithelial cells from cystic fibrosis patients and healthy subjects. Infect. Immun. *60*, 2808-2814.

Saiman, L., and Prince, A. (1993). *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J. Clin. Invest. *92*, 1875-1880.

Sanderson, P.J. (1999). Orthopaedic implant infections. Curr. Opin. Infect. Dis. *12*, 347-350.

Sandrock, R.W., and Vanetten, H.D. (1998). Fungal Sensitivity to and Enzymatic Degradation of the Phytoanticipin alpha-Tomatine. Phytopathology *88*, 137-143.

Saxild, H.H., Andersen, L.N., and Hammer, K. (1996). *dra-nupC-pdp* operon of *Bacillus subtilis*: nucleotide sequence, induction by deoxyribonucleosides, and transcriptional regulation by the *deoR*-encoded DeoR repressor protein. J. Bacteriol. *178*, 424-434.

Schaaff, F., Bierbaum, G., Baumert, N., Bartmann, P., and Sahl, H.G. (2003). Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. Int. J. Med. Microbiol. *293*, 427-435.

Schmidt, K.A., Manna, A.C., Gill, S., and Cheung, A.L. (2001). SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. Infect. Immun. *69*, 4749-4758.

Schneider, M., Muhlemann, K., Droz, S., Couzinet, S., Casaulta, C., and Zimmerli, S. (2008). Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. J. Clin. Microbiol. *46*, 1832-1834.

Schroder, A., Kland, R., Peschel, A., von Eiff, C., and Aepfelbacher, M. (2006). Live cell imaging of phagosome maturation in *Staphylococcus aureus* infected human endothelial cells: small colony variants are able to survive in lysosomes. Med. Microbiol. Immunol. *195*, 185-194.

Schulthess, B., Bloes, D.A., Francois, P., Girard, M., Schrenzel, J., Bischoff, M., and Berger-Bachi, B. (2011). The sigmaB-dependent *yabJ-spoVG* operon is involved in the regulation of extracellular nuclease, lipase, and protease expression in *Staphylococcus aureus*. J. Bacteriol. *193*, 4954-4962.

Schulthess, B., Meier, S., Homerova, D., Goerke, C., Wolz, C., Kormanec, J., Berger-Bachi, B., and Bischoff, M. (2009). Functional characterization of the sigmaB-dependent *yabJ-spoVG* operon in *Staphylococcus aureus*: role in methicillin and glycopeptide resistance. Antimicrob. Agents Chemother. *53*, 1832-1839.

Schwan, W.R., Langhorne, M.H., Ritchie, H.D., and Stover, C.K. (2003). Loss of hemolysin expression in *Staphylococcus aureus agr* mutants correlates with selective survival during mixed infections in murine abscesses and wounds. FEMS Immunol. Med. Microbiol. *38*, 23-28.

Scott, M.G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A., Wang, A., Lee, K., Doria, S., Hamill, P., *et al.* (2007). An anti-infective peptide that selectively modulates the innate immune response. Nat. Biotechnol. *25*, 465-472.

Seifert, H., Wisplinghoff, H., Schnabel, P., and von Eiff, C. (2003). Small colony variants of *Staphylococcus aureus* and pacemaker-related infection. Emerg. Infect. Dis. *9*, 1316-1318.

Sendi, P., and Proctor, R.A. (2009). *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends Microbiol. *17*, 54-58.

Senn, M.M., Bischoff, M., von Eiff, C., and Berger-Bachi, B. (2005a). sigmaB activity in a *Staphylococcus aureus hemB* mutant. J. Bacteriol. *187*, 7397-7406.

Senn, M.M., Giachino, P., Homerova, D., Steinhuber, A., Strassner, J., Kormanec, J., Fluckiger, U., Berger-Bachi, B., and Bischoff, M. (2005b). Molecular analysis and organization of the sigmaB operon in *Staphylococcus aureus*. J. Bacteriol. *187*, 8006-8019.

Shah, P.M. (2005). The need for new therapeutic agents: what is the pipeline? Clin. Microbiol. Infect. *11 Suppl 3*, 36-42.

Shinji, H., Yosizawa, Y., Tajima, A., Iwase, T., Sugimoto, S., Seki, K., and Mizunoe, Y. (2011). Role of fibronectin-binding proteins A and B in *in vitro* cellular infections and *in vivo* septic infections by *Staphylococcus aureus*. Infect. Immun. *79*, 2215-2223.

Sibley, C.D., Duan, K., Fischer, C., Parkins, M.D., Storey, D.G., Rabin, H.R., and Surette, M.G. (2008). Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. PLoS Pathog. *4*, e1000184.

Sibley, C.D., and Surette, M.G. (2011). The polymicrobial nature of airway infections in cystic fibrosis: Cangene Gold Medal Lecture. Can. J. Microbiol. *57*, 69-77.

Simons, V., Morrissey, J.P., Latijnhouwers, M., Csukai, M., Cleaver, A., Yarrow, C., and Osbourn, A. (2006). Dual effects of plant steroidal alkaloids on *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. *50*, 2732-2740.

Singh, R., Ray, P., Das, A., and Sharma, M. (2009). Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. J. Med. Microbiol. *58*, 1067-1073.

Singh, R., Ray, P., Das, A., and Sharma, M. (2010a). Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant. J. Med. Microbiol. *59*, 521-527.

Singh, R., Ray, P., Das, A., and Sharma, M. (2010b). Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J. Antimicrob. Chemother. *65*, 1955-1958.

Singh, V.K., Schmidt, J.L., Jayaswal, R.K., and Wilkinson, B.J. (2003). Impact of *sigB* mutation on *Staphylococcus aureus* oxacillin and vancomycin resistance varies with parental background and method of assessment. Int. J. Antimicrob. Agents *21*, 256-261.

Sinha, B., Francois, P.P., Nusse, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., and Krause, K.H. (1999). Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol. *1*, 101-117.

Sinha, B., and Fraunholz, M. (2010). *Staphylococcus aureus* host cell invasion and post-invasion events. Int. J. Med. Microbiol. *300*, 170-175.

Sinha, B., and Herrmann, M. (2005). Mechanism and consequences of invasion of endothelial cells by *Staphylococcus aureus*. Thromb. Haemost. *94*, 266-277.

Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'Argenio, D.A., Miller, S.I., Ramsey, B.W., Speert, D.P., Moskowitz, S.M., *et al.* (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. USA *103*, 8487-8492.

Smith, J.J., Travis, S.M., Greenberg, E.P., and Welsh, M.J. (1996). Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell *85*, 229-236.

Smith, K.M., Slugoski, M.D., Loewen, S.K., Ng, A.M., Yao, S.Y., Chen, X.Z., Karpinski, E., Cass, C.E., Baldwin, S.A., and Young, J.D. (2005). The broadly selective human Na+/nucleoside cotransporter (hCNT3) exhibits novel cation-coupled nucleoside transport characteristics. J. Biol. Chem. *280*, 25436-25449.

Somerville, G.A., Chaussee, M.S., Morgan, C.I., Fitzgerald, J.R., Dorward, D.W., Reitzer, L.J., and Musser, J.M. (2002). *Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival. Infect. Immun. *70*, 6373-6382.

Sompolinsky, D., Cohen, M., and Ziv, G. (1974). Epidemiological and biochemical studies on thiamine-less dwarf-colony variants of *Staphylococcus aureus* as etiological agents of bovine mastitis. Infect. Immun. *9*, 217-228.

Song, J., Lays, C., Vandenesch, F., Benito, Y., Bes, M., Chu, Y., Lina, G., Romby, P., Geissmann, T., and Boisset, S. (2012). The Expression of Small Regulatory RNAs in Clinical Samples Reflects the Different Life Styles of *Staphylococcus aureus* in Colonization vs. Infection. PLoS One *7*, e37294.

Song, Z., Kong, K.F., Wu, H., Maricic, N., Ramalingam, B., Priestap, H., Schneper, L., Quirke, J.M., Hoiby, N., and Mathee, K. (2010). Panax ginseng has anti-infective activity against opportunistic pathogen *Pseudomonas aeruginosa* by inhibiting quorum sensing, a bacterial communication process critical for establishing infection. Phytomedicine *17*, 1040-1046.

Spearman, P., Lakey, D., Jotte, S., Chernowitz, A., Claycomb, S., and Stratton, C. (1996). Sternoclavicular joint septic arthritis with small-colony variant *Staphylococcus aureus*. Diagn. Microbiol. Infect. Dis. *26*, 13-15.

Sriramulu, D.D., Nimtz, M., and Romling, U. (2005). Proteome analysis reveals adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment. Proteomics *5*, 3712-3721.

Stewart, P.S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. *292*, 107-113.

Stoodley, P., Sauer, K., Davies, D.G., and Costerton, J.W. (2002). Biofilms as complex differentiated communities. Annu. Rev. Microbiol. *56*, 187-209.

Talbot, G.H., Bradley, J., Edwards, J.E., Jr., Gilbert, D., Scheld, M., and Bartlett, J.G. (2006). Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin. Infect. Dis. *42*, 657-668.

Tamber, S., Schwartzman, J., and Cheung, A.L. (2010). Role of PknB kinase in antibiotic resistance and virulence in community-acquired methicillin-resistant *Staphylococcus aureus* strain USA300. Infect. Immun. *78*, 3637-3646.

Tegmark, K., Karlsson, A., and Arvidson, S. (2000). Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. *37*, 398-409.

Teichgraber, V., Ulrich, M., Endlich, N., Riethmuller, J., Wilker, B., De Oliveira-Munding, C.C., van Heeckeren, A.M., Barr, M.L., von Kurthy, G., Schmid, K.W., *et al.* (2008). Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nat. Med. *14*, 382-391.

Throup, J.P., Zappacosta, F., Lunsford, R.D., Annan, R.S., Carr, S.A., Lonsdale, J.T., Bryant, A.P., McDevitt, D., Rosenberg, M., and Burnham, M.K. (2001). The *srhSR* gene pair from *Staphylococcus aureus*: genomic and proteomic approaches to the identification and characterization of gene function. Biochemistry *40*, 10392-10401.

Tirouvanziam, R., de Bentzmann, S., Hubeau, C., Hinnrasky, J., Jacquot, J., Peault, B., and Puchelle, E. (2000). Inflammation and infection in naive human cystic fibrosis airway grafts. Am. J. Respir. Cell Mol. Biol. *23*, 121-127.

Tirouvanziam, R., Khazaal, I., and Peault, B. (2002). Primary inflammation in human cystic fibrosis small airways. Am. J. Physiol. Lung Cell. Mol. Physiol. *283*, L445-451.

Toledo-Arana, A., Merino, N., Vergara-Irigaray, M., Debarbouille, M., Penades, J.R., and Lasa, I. (2005). *Staphylococcus aureus* develops an alternative, *ica*-independent biofilm in the absence of the *arlRS* two-component system. J. Bacteriol. *187*, 5318-5329.

Tosi, M.F., Zakem, H., and Berger, M. (1990). Neutrophil elastase cleaves C3bi on opsonized pseudomonas as well as CR1 on neutrophils to create a functionally important opsonin receptor mismatch. J. Clin. Invest. *86*, 300-308.

Tuchscherr, L., Heitmann, V., Hussain, M., Viemann, D., Roth, J., von Eiff, C., Peters, G., Becker, K., and Loffler, B. (2010). *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J. Infect. Dis. *202*, 1031-1040.

Tuchscherr, L., Medina, E., Hussain, M., Volker, W., Heitmann, V., Niemann, S., Holzinger, D., Roth, J., Proctor, R.A., Becker, K., *et al.* (2011). *Staphylococcus aureus* phenotype

switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol. Med. *3*, 129-141.

Ulrich, M., Bastian, M., Cramton, S.E., Ziegler, K., Pragman, A.A., Bragonzi, A., Memmi, G., Wolz, C., Schlievert, P.M., Cheung, A., *et al.* (2007). The staphylococcal respiratory response regulator SrrAB induces *ica* gene transcription and polysaccharide intercellular adhesin expression, protecting *Staphylococcus aureus* from neutrophil killing under anaerobic growth conditions. Mol. Microbiol. *65*, 1276-1287.

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J.M., Amorena, B., Penades, J.R., and Lasa, I. (2003). SarA and not sigmaB is essential for biofilm development by *Staphylococcus aureus*. Mol. Microbiol. *48*, 1075-1087.

Valle, J., Vergara-Irigaray, M., Merino, N., Penades, J.R., and Lasa, I. (2007). sigmaB regulates IS256-mediated *Staphylococcus aureus* biofilm phenotypic variation. J. Bacteriol. *189*, 2886-2896.

van Belkum, A., Melles, D.C., Nouwen, J., van Leeuwen, W.B., van Wamel, W., Vos, M.C., Wertheim, H.F., and Verbrugh, H.A. (2009). Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. Infect. Genet. Evol. *9*, 32-47.

Vaudaux, P., Francois, P., Bisognano, C., Kelley, W.L., Lew, D.P., Schrenzel, J., Proctor, R.A., McNamara, P.J., Peters, G., and Von Eiff, C. (2002). Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. Infect. Immun. *70*, 5428-5437.

Venkatakrishnan, A., Stecenko, A.A., King, G., Blackwell, T.R., Brigham, K.L., Christman, J.W., and Blackwell, T.S. (2000). Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. *23*, 396-403.

Verkman, A.S., Song, Y., and Thiagarajah, J.R. (2003). Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. Am. J. Physiol. Cell.Physiol. *284*, C2-15.

Vesga, O., Groeschel, M.C., Otten, M.F., Brar, D.W., Vann, J.M., and Proctor, R.A. (1996). *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. J. Infect. Dis. *173*, 739-742.

Vij, N., Mazur, S., and Zeitlin, P.L. (2009). CFTR is a negative regulator of NFkappaB mediated innate immune response. PLoS One *4*, e4664.

Voggu, L., Schlag, S., Biswas, R., Rosenstein, R., Rausch, C., and Gotz, F. (2006). Microevolution of cytochrome bd oxidase in Staphylococci and its implication in resistance to respiratory toxins released by *Pseudomonas*. J. Bacteriol. *188*, 8079-8086.

Vojtov, N., Ross, H.F., and Novick, R.P. (2002). Global repression of exotoxin synthesis by staphylococcal superantigens. Proc. Natl. Acad. Sci. USA *99*, 10102-10107.

von Eiff, C., Bettin, D., Proctor, R.A., Rolauffs, B., Lindner, N., Winkelmann, W., and Peters, G. (1997a). Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. Clin. Infect. Dis. *25*, 1250-1251.

von Eiff, C., Heilmann, C., Proctor, R.A., Woltz, C., Peters, G., and Gotz, F. (1997b). A site-directed *Staphylococcus aureus hemB* mutant is a small-colony variant which persists intracellularly. J. Bacteriol. *179*, 4706-4712.

von Eiff, C., McNamara, P., Becker, K., Bates, D., Lei, X.H., Ziman, M., Bochner, B.R., Peters, G., and Proctor, R.A. (2006a). Phenotype microarray profiling of *Staphylococcus aureus menD* and *hemB* mutants with the small-colony-variant phenotype. J. Bacteriol. *188*, 687-693.

von Eiff, C., Peters, G., and Becker, K. (2006b). The small colony variant (SCV) concept -- the role of staphylococcal SCVs in persistent infections. Injury *37 Suppl 2*, S26-33.

von Gotz, F., Haussler, S., Jordan, D., Saravanamuthu, S.S., Wehmhoner, D., Strussmann, A., Lauber, J., Attree, I., Buer, J., Tummler, B., *et al.* (2004). Expression analysis of a highly adherent and cytotoxic small colony variant of *Pseudomonas aeruginosa* isolated from a lung of a patient with cystic fibrosis. J. Bacteriol. *186*, 3837-3847.

von Kockritz-Blickwede, M., Rohde, M., Oehmcke, S., Miller, L.S., Cheung, A.L., Herwald, H., Foster, S., and Medina, E. (2008). Immunological mechanisms underlying the genetic predisposition to severe *Staphylococcus aureus* infection in the mouse model. Am. J. Pathol. *173*, 1657-1668.

Wagner, V.E., and Iglewski, B.H. (2008). *P. aeruginosa* Biofilms in CF Infection. Clin. Rev. Allergy Immunol. *35*, 124-134.

Wang, B., Yurecko, R.S., Dedhar, S., and Cleary, P.P. (2006). Integrin-linked kinase is an essential link between integrins and uptake of bacterial pathogens by epithelial cells. Cell Microbiol. *8*, 257-266.

Wang, J.H., Zhou, Y.J., and He, P. (2010). *Staphylococcus aureus* induces apoptosis of human monocytic U937 cells via NF-kappaB signaling pathways. Microb. Pathog. *49*, 252-259.

Weaver, V.B., and Kolter, R. (2004). *Burkholderia* spp. alter *Pseudomonas aeruginosa* physiology through iron sequestration. J. Bacteriol. *186*, 2376-2384.

Weber, A.J., Soong, G., Bryan, R., Saba, S., and Prince, A. (2001). Activation of NF-kappaB in airway epithelial cells is dependent on CFTR trafficking and Cl- channel function. Am. J. Physiol. Lung Cell. Mol. Physiol. *281*, L71-78.

Weidenmaier, C., Peschel, A., Xiong, Y.Q., Kristian, S.A., Dietz, K., Yeaman, M.R., and Bayer, A.S. (2005). Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. J. Infect. Dis. *191*, 1771-1777.

Wenzel, R., Bate, G., and Kirkpatrick, P. (2005). Tigecycline. Nat. Rev. Drug Discov. *4*, 809-810.

Werbick, C., Becker, K., Mellmann, A., Juuti, K.M., von Eiff, C., Peters, G., Kuusela, P.I., Friedrich, A.W., and Sinha, B. (2007). Staphylococcal chromosomal cassette *mec* type I, *spa* type, and expression of Pls are determinants of reduced cellular invasiveness of methicillin-resistant *Staphylococcus aureus* isolates. J. Infect. Dis. *195*, 1678-1685.

Witte, W., Cuny, C., Klare, I., Nubel, U., Strommenger, B., and Werner, G. (2008). Emergence and spread of antibiotic-resistant Gram-positive bacterial pathogens. Int. J. Med. Microbiol. *298*, 365-377.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K.C., Birrer, P., Bellon, G., Berger, J., Weiss, T., *et al.* (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas infections* of cystic fibrosis patients. J. Clin. Invest. *109*, 317-325.

Wright, J.S., 3rd, Jin, R., and Novick, R.P. (2005). Transient interference with staphylococcal quorum sensing blocks abscess formation. Proc. Natl. Acad. Sci. USA *102*, 1691-1696.

Wu, H., Lee, B., Yang, L., Wang, H., Givskov, M., Molin, S., Hoiby, N., and Song, Z. (2011). Effects of ginseng on *Pseudomonas aeruginosa* motility and biofilm formation. FEMS Immunol. Med. Microbiol. *62*, 49-56.

Xu, K.D., McFeters, G.A., and Stewart, P.S. (2000). Biofilm resistance to antimicrobial agents. Microbiology *146 (Pt 3)*, 547-549.

Yang, L., Liu, Y., Markussen, T., Hoiby, N., Tolker-Nielsen, T., and Molin, S. (2011). Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. FEMS Immunol. Med. Microbiol.

Yarwood, J.M., McCormick, J.K., and Schlievert, P.M. (2001). Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. J. Bacteriol. *183*, 1113-1123.

Yarwood, J.M., and Schlievert, P.M. (2003). Quorum sensing in *Staphylococcus* infections. J. Clin. Invest. *112*, 1620-1625.

Zar, H., Saiman, L., Quittell, L., and Prince, A. (1995). Binding of *Pseudomonas aeruginosa* to respiratory epithelial cells from patients with various mutations in the cystic fibrosis transmembrane regulator. J. Pediatr. *126*, 230-233.

Zhu, L., Inoue, K., Yoshizumi, S., Kobayashi, H., Zhang, Y., Ouyang, M., Kato, F., Sugai, M., and Inouye, M. (2009). *Staphylococcus aureus* MazF specifically cleaves a pentad sequence, UACAU, which is unusually abundant in the mRNA for pathogenic adhesive factor SraP. J. Bacteriol. *191*, 3248-3255.