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GÉNOMIQUE FONCTIONNELLE DE *PSEUDOMONAS AERUGINOSA* ET ANALYSE MOLÉCULAIRE FINE D'UN FACTEUR SIGMA-ANTI-SIGMA.

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Résumé

Pseudomonas aeruginosa est un pathogène opportuniste pouvant causer des infections pulmonaires chroniques chez les gens atteints de la Fibrose Kystique (FK). Pour réussir à s'implanter dans les voies respiratoires des patients FK, *P. aeruginosa* dispose d'un important arsenal de facteurs de virulence, dont la sécrétion de protéases, de lipases, de phospholipases ainsi que la production de toxines spécifiques. Le séquençage complet du chromosome de *P. aeruginosa* souche PAO1 (6.3 Mb) a révélé une organisation génomique hautement régulée. Dans le but de mieux comprendre l'interaction hôte-pathogène, nous avons utilisé la technique de mutagenèse à étiquette (STM). La STM a permis d'isoler 214 mutants incapables de maintenir l'infection pulmonaire chronique chez le rat. Parmi ceux-ci, le mutant STM2895, contenant un transposon dans le gène PA2895, a été retenu pour des analyses plus approfondies. L'étude phénotypique de STM2895 a permis de constater un défaut dans la production d'exoprotéases lorsque comparé à la souche sauvage PAO1. La caractérisation biochimique de ce défaut, utilisant des tests de dégradation spécifiques et l'immunobuvardage, a démontré qu'au moins deux des quatre protéases majeures sécrétées par *P. aeruginosa* sont impliquées. En effet, les élastases LasA et LasB ont été démontrées non fonctionnelles probablement dues à un problème de repliement. PA2895, une protéine possédant un domaine transmembranaire prédit, ne code pour aucune fonction connue, mais il est co-transcrit avec le gène PA2896, un facteur sigma putatif de type ECF (extracytoplasmic function). Des analyses en transcriptome sur le mutant STM2895, ainsi que sur un mutant de délétion de PA2896, ont permis de lier l'opéron au métabolisme du fer. De plus, des études *in vivo* dans le modèle d'infection pulmonaire chronique chez le rat ont clairement démontré que le mutant STM2895 est incapable de se maintenir dans l'hôte au même niveau que la souche sauvage PAO1. Le gène PA2895 est donc essentiel au maintien *in vivo* de *P. aeruginosa* dans le poumon de rat.

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that can cause pulmonary infections in cystic fibrosis patients (CF). To overcome innate self defense, *P. aeruginosa* possesses a wide arsenal of virulence factors. These include degradation enzymes such as proteases, lipases and phospholipases and the production of three specific toxins: exotoxin A and exoenzymes S and T. Sequencing of the complete *P. aeruginosa* chromosome (strain PAO1) of 6.3 Mb revealed a highly regulated and complex genomic organization. In order to better understand host-pathogen molecular interactions, we developped a new signature-tagged mutagenesis (STM) approach based on PCR screening. The PCR-based STM technology lead to the identification of 214 mutants deficient in their ability to maintain a chronic pulmonary infection in the rat lung. In that pool of STM mutants, STM2895, which contains a transposon insertion in functional PA2895, was the most frequently drafted during the whole mutant library screening. Phenotypic analyses of the STM2895 strain allowed us to identify an exoprotease production defect as compared with wild type strain PAO1. The biochemical characterization of that proteolytic default using specific degradation assays combined with western blotting revealed that at least two (LasA and LasB) of the four major exoproteases from *P. aeruginosa* STM2895 strain are inactive. In fact, LasA and LasB elastases were shown to be present in the STM2895 culture supernatant, correctly processed but inactive due to a probable misfolding of proteins. The PA2895 gene (unknown function) encodes a protein with a predicted transmembrane domain. Basic genomic context analyses strongly suggest a cotranscription unit with the downstream gene PA2896, a putative sigma 70 factor from ECF (extracytoplasmic function) type. Microarray experiments on the STM2895 strain and an insertional mutant of the PA2896 gene were performed to establish a link between the putative PA2895-PA2896 operon and the metabolism of iron. Transcriptome analysis also demonstrated a repressive action of PA2895 on the transcription of PA2896 putative sigma factor. Finally, *in vivo* studies in the rat lung chronic infection model clearly showed a ten-fold decrease in survival capacity of the mutant strain when compared to the PAO1 wild-type strain.

Avant-Propos

Ma thèse de doctorat comporte quatre grands chapitres. Le premier chapitre inclut une brève introduction à la biologie du pathogène opportuniste *Pseudomonas aeruginosa*. Le second chapitre est un article de revue sur les facteurs sigma de *Pseudomonas aeruginosa* soumis pour évaluation dans le journal *Molecular Microbiology*. Le troisième chapitre est un article de recherche publié dans le journal *Environmental Microbiology* traitant des résultats obtenus lors du criblage *in vivo* d'une banque de mutants obtenus via la STM (Signature-Tagged Mutagenesis). Le quatrième et dernier chapitre de ma thèse est également un article de recherche soumis pour évaluation à *Molecular Microbiology* et regroupe essentiellement les résultats issus de la caractérisation fine du mutant STM2895.

Avant de présenter mes travaux, j'aimerais d'abord adresser mes remerciements les plus sincères au Dr Roger C. Levesque pour avoir, dans un premier temps, accepter que je poursuive des études de troisième cycle au sein de son laboratoire. Dans un deuxième temps, pour m'avoir confié un projet d'intérêt en regard à la pathogénie bactérienne de *Pseudomonas aeruginosa*. Et finalement, d'avoir dirigé aussi habilement et avec tant de professionnalisme ma thèse de doctorat. Roger : "Aujourd'hui, je comprends que c'est par le travail que l'on réussit."

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«Savoir s’étonner à propos est le premier pas
fait sur la route de la découverte.»
Louis Pasteur (1822-1895)

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Chapitre 1

Le chapitre 1 constitue d'abord et avant tout une introduction générale à la biologie de *Pseudomonas aeruginosa*. Il a été écrit selon une approche logique visant à bien faire ressortir les différentes relations qui existent entre l'hôte et le pathogène lors d'une infection pulmonaire chronique par exemple. Du même coup, le chapitre 1 se complète sur une brève mise en situation du projet et de toute la rationnelle qui s'y rattache.

Présentation de *Pseudomonas aeruginosa*

Caractéristiques générales

Pseudomonas aeruginosa (*P. aeruginosa*) est un bacille à Gram-négatif, aérobie strict, non sporulant de forme droite ou légèrement courbée. Il mesure entre 1 et 5 µm de long par 0,5 à 1.0 µm de large. Plusieurs isolats ont démontré la capacité de croître en anaérobiose en utilisant le nitrate (NO_2^-) comme accepteur final d'électron. Ainsi, le nitrate est réduit en nitrite de même qu'en azote moléculaire. *P. aeruginosa* est une bactérie motile grâce à la présence d'un flagelle monotriche (un seul) polaire. Cette bactérie est catalase positive et oxydase positive. *P. aeruginosa* possède une versatilité nutritionnelle remarquable pouvant utiliser une variété de sucres simples et complexes, d'alcools et d'acides aminés comme seule source de carbone. *P. aeruginosa* est capable de se multiplier à l'intérieur d'un large spectre de température allant de 4 à 45°C. La température optimale de croissance se situe entre 30 et 37°C, ce qui en fait une bactérie mésophile. La morphologie coloniale de *P. aeruginosa*, de même que pour tout le genre *Pseudomonas*, est facilement distinctive grâce à la production de pigments verts diffusibles dans le milieu extracellulaire (Palleroni, 1984). Les différentes caractéristiques morphologiques et nutritionnelles de *P. aeruginosa* sont résumées et comparées aux *Pseudomonas spp*. au Tableau 1.

Tableau 1. Caractéristiques microbiologiques et biochimiques de quelques *Pseudomonas* spp [a]

Test	***P. aeruginosa* (n=93) [b]**	***P. fluorescens* (n=223)**	***P. putida* (n=335)**	***P. stutzeri* (n=181)**
Oxydase	100	100	100	100
Croissance :				
MacConkey	99	100	100	100
Cetrimide	88	96	93	3
6.5% NaCl	7	3	11	100
42°C	100	0	0	90
Réduction nitrates	74	19	0	100
Azote moléculaire	60	4	0	100
Pyoverdine	69	91	82	0
Arg dihydrolase	99	99	99	0
Phe déaminase	8	3	2	55
Hémolyse	38	14	0	0
Hydrolyse :				
Urée	66	44	43	17
ADN	9	0	0	0
Lécithine	8	89	0	8
Gélatine	46	100	0	0
Acétamide	37	1	3	0
Production d'acide :				
Glucose	98	100	100	100
Fructose	89	99	99	95
Galactose	81	98	99	91
Mannose	79	99	99	89
Rhamnose	22	43	28	23
Xylose	85	97	98	94
Lactose	0	11	13	0
Sucrose	0	47	10	0
Maltose	12	31	19	99
Mannitol	68	93	17	70
Lactose (10%)	14	61	42	0
Motilité	96	100	100	100
Nombre de flagelle	1	>1	>1	1

(Tableau adapté de Gilligan, 1995)

a Les nombres dans le tableau font référence aux pourcentages des souches positives pour un test donné.

b Les nombres entre parenthèses font référence au nombre d'isolats inclus dans tous les tests pour chaque souche.

Distribution environnementale

La distribution de *P. aeruginosa* est très large mais c'est d'abord et avant tout un organisme environnemental que l'on retrouve dans l'eau, le sol et sur la majorité des espèces végétales, incluant fruits et légumes. Notons entre autres : l'eau du robinet, les fluides d'humidification oculaires, les savons, les désinfectants, les onguents et cosmétiques. On le retrouve aussi dans les spas, bains thérapeutiques, ainsi que sur plusieurs surfaces humides comme les lentilles cornéennes, le pommeau de douche, les équipements de dialyse, les appareils d'assistance respiratoire et les cathéters. Sa large distribution et sa grande capacité de survie dans le milieu aqueux, font que cette bactérie est présente en milieux hospitaliers détenant ainsi une grande part des responsabilités des infections nosocomiales. À cause d'une implication clinique majeure, *P. aeruginosa* est certes le membre le plus étudié du genre *Pseudomonas* (Grundmann *et al.*, 1993).

P. aeruginosa n'est, de façon générale, pas un membre de la microflore normale humaine. L'intestin en est colonisé, mais seulement suite à l'ingestion de l'organisme. La colonisation du tractus respiratoire est commune chez les patients hospitalisés et surtout lors de l'intubation. Plus longtemps le patient sera sous assistance respiratoire, plus importante sera la colonisation (Pollack, 1990). Le fait que l'on retrouve *P. aeruginosa* en milieu hospitalier conduit souvent à des bactériémies à la suite d'une exposition prolongée ou à l'infection d'une plaie post-opératoire.

Implications cliniques

P. aeruginosa est le pathogène humain le plus important du genre *Pseudomonas* étant donné le grand nombre et type d'infections causées ainsi que la morbidité et la mortalité qui leurs sont associées (Pollack, 1990). Il est capable de combiner efficacement adaptabilité aux milieux humides et production d'un imposant arsenal de facteurs de virulence. Les affections causées vont d'une simple infection cutanée superficielle à des septicémies

fulminantes. Les infections à *P. aeruginosa* acquises en communauté chez les individus immunocompétents tendent à être localisées et sont fréquemment associées à de l'eau ou de la nourriture contaminée. Les infections causées à l'œil provoquent souvent des lésions cornéennes. Ce type d'infection est directement lié à l'usage de lentilles cornéennes. Les solutions contaminées pour lentilles ainsi que l'usage de l'eau du robinet pour l'entretien de celles-ci ont été identifiées comme sources de contamination. (Holland *et al*., 1993) Les infections oculaires à *P. aeruginosa* peuvent rapidement mener à la formation d'ulcères de la cornée et à la perte de la vue si l'infection n'est pas traitée promptement. L'infection la plus sévère pouvant être acquise en communauté est l'endocardite provenant des seringues contaminées par les usagers de drogues injectables. Les drogues injectées sont dans beaucoup de cas mélangées avec de l'eau du robinet contaminée par *P. aeruginosa* causant la bactériémie (Pollack, 1990).

On retrouve également *P. aeruginosa* sous un phénotype inhabituel que l'on nomme mucoïde chez environ 80% des gens atteints de la Fibrose Kystique (FK). Le phénotype mucoïde de *P. aeruginosa* se trouve toujours en association avec de graves infections pulmonaires chroniques. On croit que c'est l'environnement inhabituel du poumon FK qui est la cause du changement vers le phénotype mucoïde. La sécrétion d'alginate, un exopolysaccharide complexe, en combinaison avec le mucus épais et collant des voies respiratoires forme un milieu de culture idéal nécessaire à l'établissement d'infections pulmonaires chroniques. Cette barrière servira donc de protection anti-phagocytique de même que d'écran contre les antibiothérapies.

La Fibrose Kystique

Les bases moléculaires

La Fibrose Kystique (FK) est une maladie résultante de différentes mutations du gène codant pour le CFTR « Cystic Fibrosis transmembrane conductance regulator ». Le CFTR est une protéine canal à ions chlorures dépendante de l'AMPc que l'on retrouve à la surface des cellules épithéliales (Rommens *et al.*, 1989). L'inactivité du CFTR est soit liée à l'absence de production de celui-ci; soit la molécule est incapable d'atteindre son site d'action, ou soit à un défaut de fonction (Bijman *et al.*, 1987). La conséquence majeure de cette anomalie est l'affaiblissement du transport des ions chlorures à la surface de la cellule. D'autres fonctions ont été attribuées au CFTR telles que la régulation d'une panoplie d'autres canaux qui pourraient, eux aussi, jouer un rôle important dans la pathogenèse de la maladie. Ainsi, la perte d'inhibition des canaux sodiques de l'épithélium mène à l'hyper absorption sodique et favorise une osmose positive vers l'épithélium. Bien que la protéine CFTR soit exprimée dans plusieurs organes, l'effet clinique dominant de la maladie se retrouve au niveau des systèmes respiratoire, gastro-intestinal et reproducteur. De ces zones touchées, les maladies pulmonaires comptent pour plus de 90% des causes de morbidité chez les patients atteints de la FK.

Comment une mutation du CFTR conduit à une maladie respiratoire?

La caractéristique marquante du poumon FK est sa colonisation par une gamme limitée d'espèces bactériennes ainsi que d'une sévère inflammation conduite essentiellement par les neutrophiles. Ces traits apparaissent tôt dans l'évolution de la maladie et persistent dans le temps pour conduire à un état d'infection pulmonaire chronique (Koch and Hoiby, 1993). Le rôle exact de la mutation du CFTR dans la chronologie des évènements conduisant à l'infection pulmonaire chronique et aux dommages irréversibles est en partie inconnu et

très controversé. Par contre, la plupart des experts s'entendent pour dire qu'à la naissance, les poumons des bébés FK sont normaux (Chow *et al.*, 1982; Oppenheimer and Esterly, 1975). Toutefois, de récentes études sur des souris ont avancé que le CFTR pourrait avoir un rôle durant la période fœtale. Effectivement, le transfert *in utero* du gène codant pour le CFTR réduit grandement les effets gastro-intestinaux de la maladie mais ne prévient pas le développement d'une pathologie pulmonaire (Larson *et al.*, 1997). Chez l'humain FK, l'analyse en continue des voies respiratoires a révélé la présence de microzones d'inflammation dès la petite enfance sans aucune présence de pathogènes cultivables. Ces données portent à croire que la mutation du CFTR lui-même pourrait être responsable de l'état pro-inflammatoire (Abman *et al.*, 1991). En accord avec cette hypothèse, des études menées sur des voies respiratoires FK humaines à l'état fœtal ont montré un haut niveau d'expression de cytokines pro-inflammatoires ainsi qu'une présence leucocytaire accrue aux muqueuses en l'absence d'infection bactérienne (Tirouvanziam *et al.*, 2000). En opposition à la même hypothèse, des études effectuées sur des lavages broncho-alvéolaires d'enfants FK ont montré un niveau normal de cytokines sans infection bactérienne, mais le déclenchement d'une réponse inflammatoire exagérée dès que des pathogènes envahissent les voies respiratoires (Armstrong *et al.*, 1995).

Les infections bactériennes associées à la FK surviennent en bas âge et sont difficiles à enrayer. Durant la petite enfance, les infections à *Staphylococcus aureus* et à *Haemophilus influenzae* sont communes et par la suite, *P. aeruginosa* s'installe et devient le pathogène majeur. Des infections chroniques à *Pseudomonas* sont présentes dans plus de 85% des cas à l'adolescence et du même fait, la bactérie est directement responsable des dommages tissulaires des poumons causés par une réponse inflammatoire agressive. Un plus petit nombre de patients vont se voir colonisés également par d'autres espèces opportunistes comme *Burkholderia cepacia*, *Stenotrophonomas maltophilia* et certaines espèces de mycobactéries non-tuberculeuses.

L'acquisition initiale

Chez un individu sain, l'entrée de *P. aeruginosa* dans les poumons est rapidement stoppée par une variété de défenses naturelles composées essentiellement du réflexe mécanique de clairance mucociliaire et de la présence des macrophages alvéolaires. Chez les patients FK, de même que ceux qui sont sous assistance respiratoire, immunodéprimés ou avec des brûlures importantes, ces barrières de défense sont faibles et leur défaillance crée l'opportunité nécessaire à l'établissement d'une infection. Bien que peu connue, on sait que c'est durant les stades précoces de la maladie que l'adhérence à la surface cellulaire des voies respiratoires et la résistance aux défenses innées de l'individu vont survenir. Mais pourquoi ces étapes sont-elles facilitées par l'environnement des voies respiratoires FK? Plusieurs explications ont été avancées dont une composition chimique anormale du liquide de surface des voies respiratoires surtout due à l'excès d'ions chlorures. De ce fait, il s'en suivrait plusieurs conséquences directes dont le fonctionnement inefficace des cils vibratiles des voies respiratoire, une diminution de la réponse des peptides anti-microbiens, un faible niveau de phagocytose et un faible niveau de molécules de défense extracellulaires comme l'oxyde nitrique et le glutathion (Davies, 2002).

Établir une infection chronique

Dès que la bactérie a réussi à s'implanter dans les voies respiratoires, elle devra y survivre et ce malgré la présence des défenses de l'hôte, innées et acquises, et d'évènements répétés d'antibiothérapie topique et systémique. Pour réussir ceci, *P. aeruginosa* devra développer une importante gamme de stratégies immuno-évasives incluant entre autres des protéines à action extracellulaires. Des protéases, des lipases, une phospholipase C hémolytique et des exoenzymes à activité ADP-ribosylation. La sécrétion de ces produits extracellulaires sera accompagnée par d'importants changements phénotypiques dépendants du système du quorum-sensing, comme la mucosité et la formation d'un biofilm, menant à une résistance accrue aux antibiotiques.

L'Élastase B

L'élastase B (également nommée protéase LasB ou pseudolysine) est une métalloprotéase neutre nécessitant le zinc comme co-facteur pour l'activité enzymatique et le calcium comme stabilisateur (Morihara *et al.,* 1985) L'élastase B est la protéine la plus abondante du surnageant de culture ayant comme principales fonctions le recyclage protéique et la dégradation de la matrice extracellulaire. En effet, LasB dégrade efficacement les constituants majeurs de la matrice de l'épithélium pulmonaire, soient l'élastine, le collagène et la fibrine. LasB s'attaque aussi aux immunoglobulines et aux cytokines comme l'interféron gamma et au facteur nécrosant des tumeurs (TNF) (Park *et al.*, 1996). De ce fait, l'élastase B est associée aux divers types de pathologies causées par *P. aeruginosa* et pointée comme étant un facteur de virulence clé (Sawa *et al.*, 1998). LasB est initialement synthétisée sous la forme d'un précurseur contenant un domaine pré-pro-mature qui consiste en un peptide signal de 23 acides aminés, un propeptide de 174 résidus et un domaine catalytique en C-terminal de 301 acides aminés (Kessler *et al.*, 1998). Le propeptide est clivé de façon autocatalytique à l'intérieur du périplasme (McIver *et al.*, 1991). Il se forme immédiatement un complexe inactif entre le propeptide et l'enzyme repliée (Kessler and Safrin, 1994) et c'est sous cette forme que l'enzyme est sécrétée dans l'environnement extracellulaire (Braun *et al.*, 2000; Kessler *et al.*, 1998). Bien que le propeptide peut être détecté dans le surnageant de culture, il est rapidement dégradé après la sécrétion de l'élastase laissant uniquement la portion stable de l'enzyme présente à l'extérieur de la cellule.

L'Élastase A

L'élastase A (également nommée protéase staphylolytique ou staphylolysine) est également une métalloprotéase dégradant l'élastine. Cependant, à elle seule, l'élastase A ne possède pas une grande activité elastolytique, mais elle agirait plutôt en synergie avec LasB, augmentant ainsi le pouvoir de dégradation (Kessler *et al.*, 1997). Contrairement à

l'élastase B, la protéase LasA possède un site de clivage complexe. Effectivement, la protéase coupe le pont peptidique suivant un couple de glycine et plus spécialement lorsque le troisième acide aminé est une autre glycine, une alanine ou une phénylalanine (Kessler *et al.*, 1997). En revanche, l'élastase A possède une importante activité staphylolytique résultant du clivage du pont pentapeptidique Gly_5 que l'on retrouve dans la structure du peptidoglycane de *Staphylococcus aureus* (Kessler *et al.*, 1993). L'élastase A est également produite, comme décrit plus haut pour l'élastase B, en pré-pro-enzyme qui devra suivre des étapes de clivages et de repliements successifs avant d'être correctement activée dans le milieu extracellulaire (Kessler *et al*., 1998).

Sécrétion d'exotoxines

La virulence de *P. aeruginosa* repose également sur la sécrétion d'exotoxines via le système de sécrétion de type III (TTSS) dont les multiples composantes sont hautement similaires à celui retrouvé sur le plasmide de virulence de *Yersinia pestis* (Hueck, 1998). Le TTSS de *P. aeruginosa*, aussi connu sous le nom de régulon de l'exoenzyme S, comporte quatre protéines effectrices : ExoS, ExoT, ExoU et ExoY. L'exoenzyme S (ExoS) et l'exoenzyme T (ExoT) possèdent toutes deux une activité d'ADP-ribosylation commune aux protéines de faible poids moléculaire de la famille Ras et liant le GTP (McGuffie *et al.*, 1998). L'exoenzyme Y (ExoY) est une adénylate cyclase dont l'activité est associée à des changements morphologiques majeurs des cellules épithéliales (Yahr *et al.*, 1998). L'exoenzyme U (ExoU) a démontré une activité lipase *in vitro* (Sato *et al.*, 2003). La production de ces exoenzymes est principalement régulée via l'activateur transcriptionnel ExsA en réponse à différents stimuli environnementaux, comme une baisse de calcium ainsi qu'un contact direct avec des cellules en cultures (Hornef *et al.*, 2000).

Un autre facteur de virulence important de *P. aeruginosa* est l'exotoxine A. L'exotoxine A possède également une activité d'ADP-ribosylation qui agit directement sur le facteur II d'élongation eucaryote, inhibant ainsi la synthèse protéique de la cellule cible (Iglewski and Kabat, 1975). La régulation du niveau d'expression de l'exotoxine A est complexe et

requiert une cascade de régulation qui résulte en un niveau maximal d'expression dans des conditions où le fer est limité (Vasil and Ochsner, 1999).

L'acquisition du fer

Le fer est un élément essentiel à la croissance de tous les organismes vivants, mais dans la majorité des milieux, la concentration du fer bio-disponible (10-7M) est largement en deçà des niveaux requis pour la croissance des bactéries comme *P. aeruginosa* (Ratledge and Dover, 2000). Les bactéries ont évolué en développant maintes stratégies en vue d'acquérir, transporter et rendre soluble le fer (de Fe^{2+} vers Fe^{3+}) (Guerinot, 1994). La méthode la plus répandue d'acquisition du fer est la production de composés hautement affin pour celui-ci: les sidérophores. Après sa sécrétion, le sidérophore chélate le fer dans l'environnement extracellulaire. Le complexe moléculaire résultant, le ferri-sidérophore, est ensuite transporté à l'intérieur du cytoplasme via des récepteurs membranaires spécifiques pour ces complexes. *P. aeruginosa* produit un sidérophore majeur appelé pyoverdine. La pyoverdine chargée de fer est transportée via le récepteur membranaire FpvA. La pyoverdine peut également agir comme molécule de signalisation induisant la sécrétion de deux autres facteurs de virulence : l'exotoxine A et l'endoprotéase PrpL (ou protéase IV). L'interaction de la ferri-pyoverdine avec FpvA transduit un signal trans-périplasmique via FpvR, un facteur anti-sigma (voir le chapitre 2 pour une description détaillée des facteurs anti-sigma), qui à son tour provoque l'activation du facteur sigma de type ECF, PvdS, le régulateur transcriptionnel clé du métabolisme du fer chez *P. aeruginosa* (Beare *et al.*, 2003). La figure 1 illustre bien l'acquisition du fer chez *P. aeruginosa* via la sécrétion de la pyoverdine.

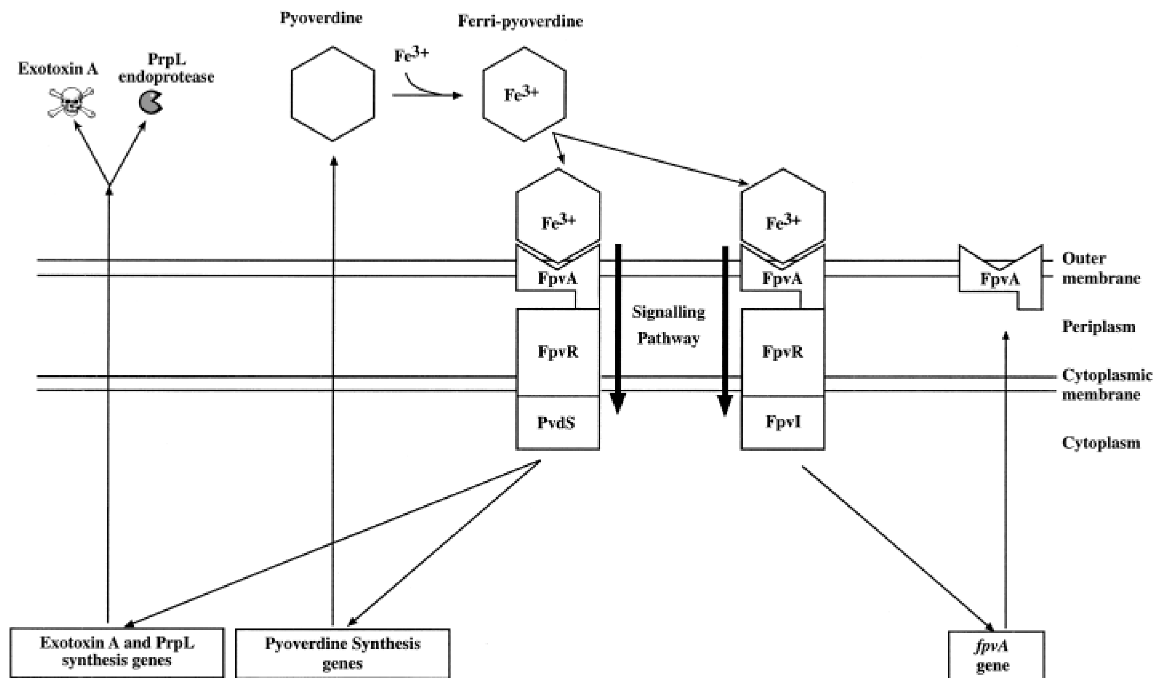

Figure 1. Modèle de transduction du signal pour l'acquisition du fer via la ferri-pyoverdine. (Tirée de Beare *et al*., 2003)

La production d'alginate

Il est connu depuis de nombreuses années que les isolats pulmonaires de *P. aeruginosa* sont très muqueux. Suivant la colonisation du poumon FK par *P. aeruginosa*, le phénotype non-mucoïde est invariablement remplacé par le phénotype mucoïde, qui est difficile à éradiquer (Doggett, 1969). La cause de cette mucosité réside dans le fait que *P. aeruginosa* sécrète un exopolysaccharide en grande quantité nommé : alginate. Physiquement, l'alginate est responsable de la neutralisation des processus immuns de l'hôte comme la phagocytose et l'opsonisation et contribue au ralentissement de la diffusion des radicaux libres comme l'oxyde nitrique (Baltimore and Mitchell, 1982 ; Simpson *et al*., 1988). L'alginate est un co-polymère, sans répétition, de ß-D-mannuronate (M) et de son C-5 épimère, le α-L-guluronate (G), relié par un pont glycosidique 1 → 4 (Evans and Linker, 1973). La suite des résidus M et G à l'intérieur du polymère conférera les différentes

propriétés physiques du gel comme son élasticité et sa viscosité. Chez *P. aeruginosa*, l'alginate est surtout divisé en bloc qui regrouperont des parties uniquement composées de M, d'autres uniquement de G et finalement, des régions mixtes M et G. Ces différents blocs sont assemblés de manière linéaire et totalement aléatoire (Chitnis and Ohman, 1990). (Figure 2) La production d'alginate est sous le contrôle du facteur sigma alternatif, σ^{22}, codé par *algT* (Voir chapitre 2). Brièvement, c'est une mutation du facteur anti-sigma Muc A, libérant AlgT, qui force une transcription non-contrôlée des gènes de biosynthèse de l'alginate (Malhotra *et al.*, 2000).

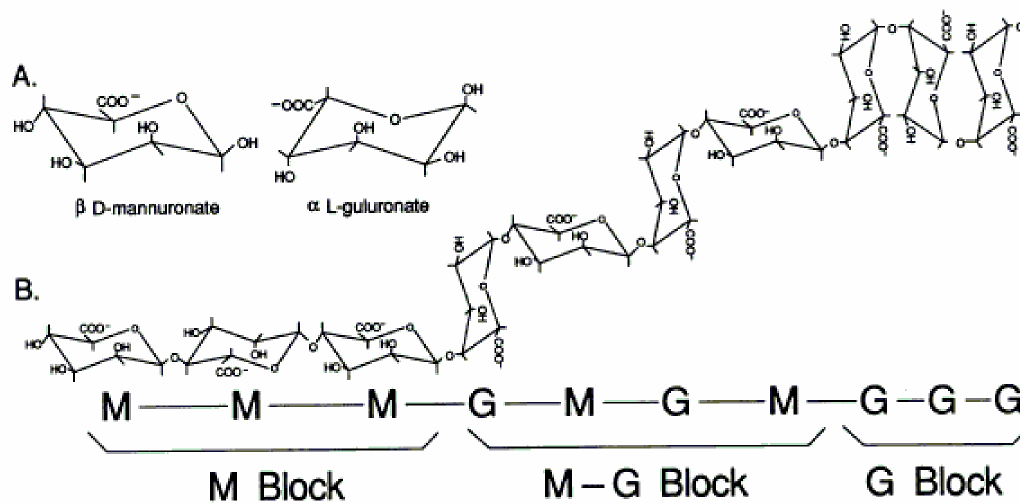

Figure 2. Structure moléculaire de l'alginate. Les deux sous-unités glucidiques, le D-mannuronate (M) et le L-guluronate (G), qui forment le polymère (A). L'assemblage du polymère linéaire d'alginate chez *P. aeruginosa* via la juxtaposition de blocs : un bloc de M au début de l'assemblage, suivi d'une région mixte M et G puis un bloc de G vers la fin de l'assemblage. (Chitnis and Ohman, 1990)

Le quorum-sensing

Chez *Pseudomonas aeruginosa*, l'orchestration des facteurs de virulence est contrôlée via un système bien connu que l'on nomme quorum sensing (QS). Le QS est sans contredits un mécanisme de régulation clé de la pathogénicité de *P. aeruginosa* permettant d'importants changements phénotypiques visant la colonisation de l'hôte (Parsek and Greenberg, 2000). Le système repose sur la diffusion et la détection de petites molécules produites dans l'environnement, les homosérines lactones (HSL). C'est la concentration des HSL retrouvées dans un milieu donné, directement liée à la densité cellulaire, qui permettra l'activation en cascade de deux systèmes inductibles de type Lux R et Lux I. Les systèmes Las et Rhl dont l'activation repose sur la concentration des molécules 3-oxo-C12-HSL et C4-HSL respectivement, sont le point de départ du mode virulence de *P. aeruginosa* (Figure 3) (Latifi *et al.*, 1996; Pesci *et al.*, 1999). Des études de transcriptome des gènes contrôlés par les circuits LasIR (dépendant de la molécule 3-oxo-C12-HSL) et RhlIR (dépendant de la molécule C4-HSL) du QS ont mis en évidence trois classes de gènes. La première classe comprend les gènes qui répondent à seulement un auto-inducteur, la deuxième regroupe ceux qui peuvent être activés par une voie ou l'autre et la dernière classe inclut ceux qui nécessitent l'activation des deux voies (Schuster *et al.*, 2003; Wagner *et al.*, 2004). De plus, les analyses de la transcription à l'aide de puces à ADN ont démontré que ces classes de gènes sont exprimées à des niveaux différents dépendamment de la phase de croissance de la culture. Ceci indique que l'architecture en tandem de ces deux réseaux nécessite une séquence d'événements coordonnés dans le temps incluant une expression transitoire de certains gènes pour l'implantation d'une infection fructueuse (Schuster *et al.*, 2003; Whiteley *et al.*, 1999).

En plus de 3-oxo-C12-HSL et de C4-HSL, *P. aeruginosa* sécrète le milieu extracellulaire du 4-quinolone comme autre molécule du QS. La synthèse et l'activité biologique de celle-ci ont été rapportées par la littérature comme étant liées aux systèmes *las* et *rhl*. La composition chimique exacte de cette molécule est 2-heptyl-3-hydroxy-4(1H)-quinolone que l'on nomme «Pseudomonas Quinolone Signal» (PQS) (Pesci *et al.*, 1999) (figure 3). Il a été démontré que LasR régule la production de PQS induisant ainsi l'expression de *lasB*,

rhlI et *rhlR* (McKnight *et al.*, 2000; Pesci *et al.*, 1999) suggérant que l'activité dépendante du PQS constitue un lien de régulation important entre *las* et *rhl*. Toutefois, McKnight *et al.* 2000 proposent que le PQS n'est pas impliqué dans le contrôle de la densité cellulaire, mais plutôt dans le cycle de croissance, une caractéristique typique des molécules du QS. (Voir la figure 4 qui résume la voie de régulation du QS)

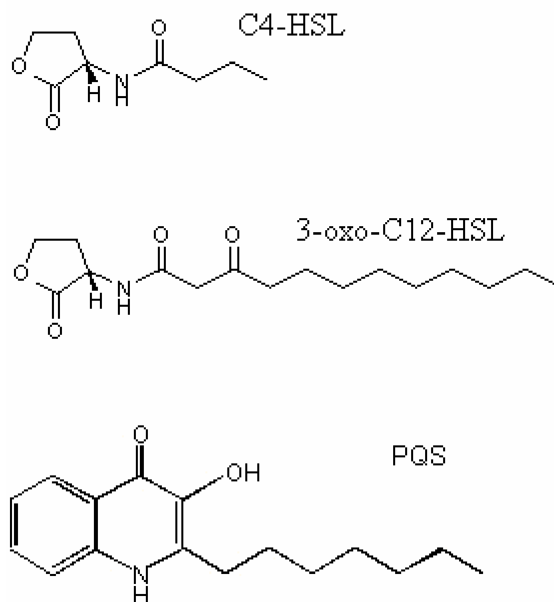

Figure 3. Composition chimique des acyl-homosérines lactones (HSL) de *P. aeruginosa*, le 3-oxo-C12-HSL et le C4-HSL, de même que celle du Pseudomonas Quinolone Signal (PQS). (Figure adaptée de http://www.nottingham.ac.uk/quorum/index.htm)

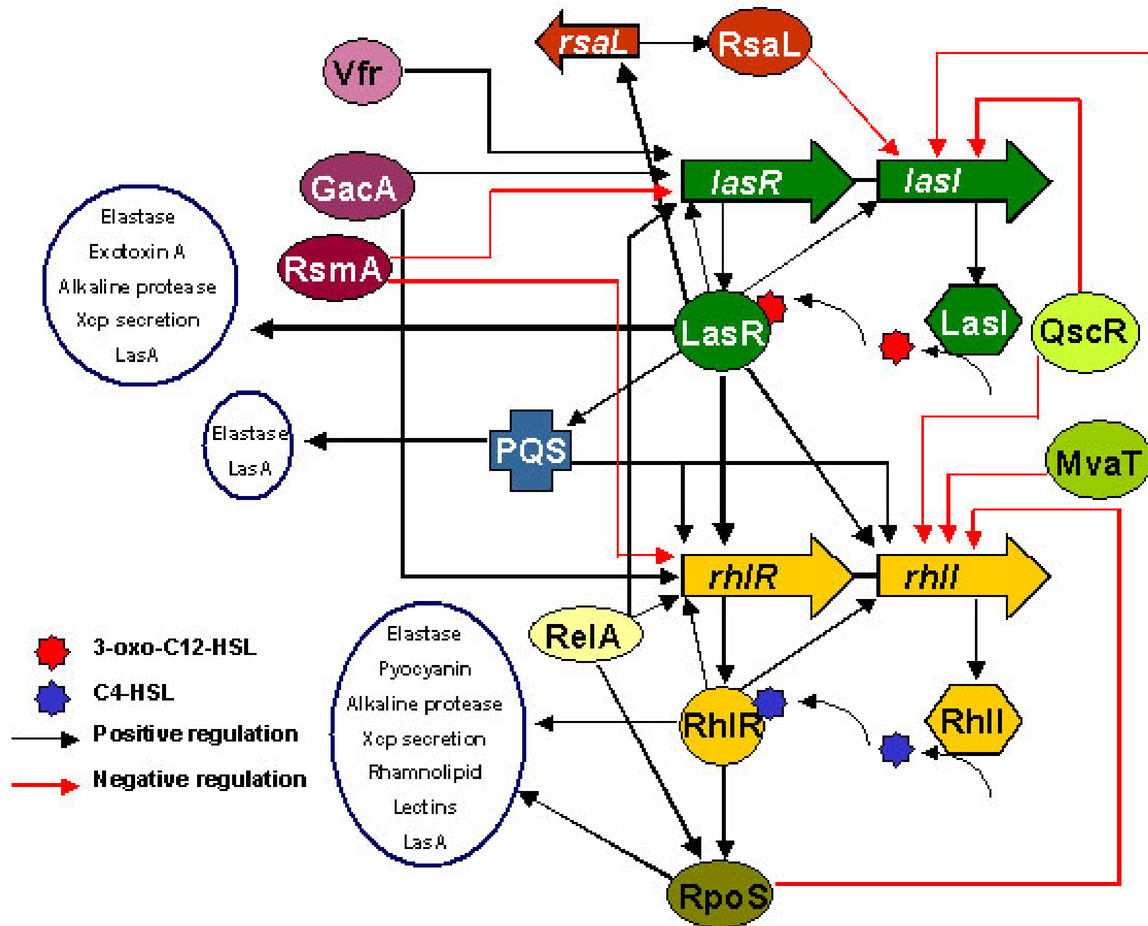

Figure 4. Schéma récapitulatif de la hiérarchie régulatrice des systèmes du «quorum-sensing» (QS) chez *P. aeruginosa*. (Figure adaptée de http://www.nottingham.ac.uk/quorum/index.htm).

La formation du biofilm

Il a été démontré que l'établissement d'un biofilm bactérien à l'intérieur des voies respiratoires des patients FK est une étape critique dans le changement phénotypique de *P. aeruginosa* vers un mode virulent. Les micro-organismes favorisent en général un mode de vie où la population cellulaire se retrouve fixée à un support (état sessile) plutôt que libre dans le milieu environnemental (état planctonique). L'attachement à une surface permet à la bactérie de s'installer et de coloniser un environnement. Après attachement sur un support, les bactéries vont mettre en place et développer une communauté organisée à laquelle on a attribué le nom de « biofilm » (Costerton *et al.*, 1999). Le biofilm se définit donc comme une population bactérienne adhérée à une surface et enrobée d'une matrice d'exopolysaccharide. La première étape de l'attachement fait intervenir les structures de locomotion comme les flagelles et les pilis de type IV (O'Toole and Kolter, 1998). Cette approche mène à un attachement transitoire. En deuxième lieu, une association stable avec la surface ou avec d'autres micro-organismes s'établit. Ces rassemblements de bactéries conduisent à la formation de micro-colonies dont la différenciation mène à l'élaboration du biofilm. La matrice d'exopolysaccharide de *P. aeruginosa* est essentiellement composée d'alginate et représente environ 85% du volume total. Cette matrice renforce la structure du biofilm tout en lui conservant une grande élasticité. À l'intérieur du biofilm, les micro-colonies sont séparées par un réseau de canaux circulant permettant, d'une part, d'acheminer l'oxygène et les nutriments à l'intérieur du biofilm, et, d'autre part, d'évacuer les déchets. Les molécules solubles peuvent diffuser à travers la matrice d'exopolysaccharide et être utilisées par les cellules. Un gradient de nutriments et d'oxygène est observable depuis le sommet du biofilm jusqu'à sa base où l'anaérobiose y règne. Ces observations sont en accord avec l'idée selon laquelle l'état métabolique d'une bactérie à l'intérieur d'un biofilm dépend de sa localisation au sein de la structure (Ramsey and Whiteley, 2004). La figure 5 illustre bien le cycle de formation d'un biofilm chez *P. aeruginosa*.

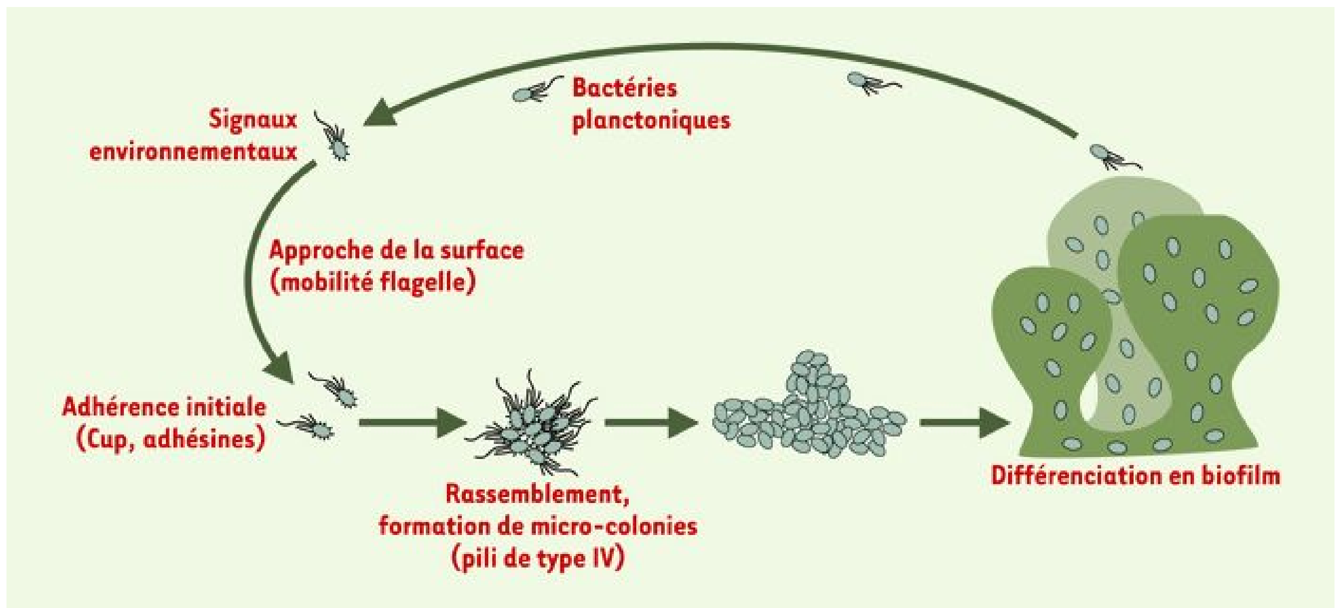

Figure 5. Cycle de formation du biofilm bactérien chez *P. aeruginosa*. (Figure tirée de Filloux et Vallet, 2003)

Résistance aux antibiotiques

Il est maintenant bien connu que *P. aeruginosa*, de même que les autres bactéries à Gram-négatif, possède une résistance intrinsèque aux antibiotiques. Cette résistance est entre autre due à la restriction des imports à travers la membrane externe, de même que des systèmes secondaires d'efflux dépendants d'énergie et de ß-lactamases (Hancock, 1997) Donc, l'importance d'une perméabilité restreinte au niveau de la membrane externe est claire car l'utilisation d'agents qui brisent la barrière externe (comme les peptides cationiques) ou des mutations qui créent de larges pores rendent les cellules beaucoup plus susceptibles à l'antibiothérapie (Huang and Hancock, 1996). La masse d'information récemment révélée sur les systèmes à efflux de *P. aeruginosa* a permis de conclure qu'ils constituaient l'élément clé de la grande résistance intrinsèque aux drogues de cette bactérie. Toutefois, il n'existe pas de différences majeures au niveau des mécanismes d'action ou de l'efficacité des systèmes à efflux de *P. aeruginosa* (comparée à *Escherichia coli* par exemple)

(Nikaido, 1994). C'est plutôt la différence de perméabilité membranaire de *P. aeruginosa* chiffrée entre 10 fois et 100 fois plus basse que les autres Gram-négatif (Nikaido and Hancock, 1986). Il est à noter toutefois que la faible perméabilité de la membrane externe de *P. aeruginosa* n'est pas le seul facteur à considérer (Nikaido, 1994). En effet, elle sert également à ralentir l'entrée des drogues pour faciliter l'action des mécanismes de résistance secondaires tels que l'efflux et la dégradation de ces substances.

La membrane extracellulaire des bactéries à Gram-négatif est constituée d'une bicouche phospholipidique, de lipopolysaccharides (LPS) et de protéines qu'on nomme porines. Les porines forment des canaux permettant la diffusion des molécules hydrophiles. OprF est sans contredit la porine majeure de *P. aeruginosa* et facilite la diffusion de grosses molécules comme des di- ou tri-saccharides et possiblement des antibiotiques (Bellido *et al.*, 1992). Deux systèmes à efflux ont été décrits comme jouant un rôle dans la résistance intrinsèque de la bactérie aux antibiotiques basée sur une expression constitutive et par l'influence de mutations nulles et/ou inhibitrices. Le premier système est celui de MexAB-OprM (Zhao *et al.*, 1998). Ce système est un prototype du système RND (resistance-nodulation-division) contenant une pompe cytoplasmique, MexB, une protéine périplasmique de liaison, MexA et une protéine de la membrane externe (OMP) OprM. Des mutations affectant n'importe quelle de ces protéines résultent en une hypersensibilité aux quinolones, tétracyclines, chloramphénicol, sulfamethoxazole, triméthoprime et quelques ß-lactames mais pas aux aminoglycosides, érythromycine et polymixines (Nikaido, 1994; Zhao *et al.*, 1998). D'autre part, le second opéron de pompe à efflux, MexX-MexY, en collaboration apparente avec OprM a été récemment impliqué dans la résistance intrinsèque aux aminoglycosides et à l'érythromycine (Aires *et al*., 1999; Westbrock-Wadman *et al*., 1999).

La mise en situation du projet

Comme discuté ci-haut, l'établissement d'une infection opportuniste à *P. aeruginosa* est multifactorielle. L'absence de traitements efficaces contre la FK, la résistance accrue aux antibiotiques et le peu d'informations disponibles concernant les caractéristiques *in vivo* d'une infection pulmonaire chronique font ressortir les besoins de nouvelles pistes de recherche pour développer des outils thérapeutiques efficaces et enrayer les problèmes d'infection persistante dus à *P. aeruginosa.* Ainsi nous croyons que l'identification de gènes essentiels à l'établissement d'une infection *in vivo* pourrait permettre de répondre en partie à la problématique soulevée. Nous soumettons l'hypothèse que les informations récentes issues du séquençage génomique de la souche PAO1 couplées aux nouvelles technologies de mutagenèse en masse permettront l'identification de nouveaux gènes impliqués dans la pathogénie de la bactérie. Ces protéines ayant une fonction inconnue à ce jour fourniront ainsi une meilleure compréhension de l'orchestration génique des facteurs de virulence proposant de nouvelles pistes thérapeutiques. L'objectif général de cette présente thèse est donc d'identifier des gènes *in vivo* essentiels à la pathogénie et au maintient de *P. aeruginosa* utilisant une technologie de mutagenèse par étiquette dans un modèle d'infection pulmonaire chronique chez le rat.

Dans le but d'identifier de nouveaux gènes associés à l'infection *in vivo* chez *P. aeruginosa*, la technologie de la STM (Signature-Tagged Mutagenesis) a été utilisée. La STM consiste en une sélection négative de mutants transpositionels atténués en virulence après passage dans un animal. Le modèle choisi pour le criblage de la banque de mutants construite est l'infection pulmonaire chronique chez le rat utilisant les billes d'agar (Cash *et al.*, 1979). Une sélection négative par PCR, des mutants récupérés dans les poumons du rat, a permis d'identifier ceux-ci comme atténués dans le maintien de l'infection. Les informations tirées de la séquence du génome de *P. aeruginosa* ont servi à cibler de façon précise les gènes touchés par les mutations. Étant donné que moins de 7% des gènes de *P. aeruginosa* ont une fonction connue (Stover *et al.*, 2000), des études de bioinformatique ont été faite sur chaque mutant STM dont le gène a été cloné et séquencé dans le but d'en connaître l'organisation génique, la fonction probable attribuée à la protéine, les

homologues connus chez d'autres espèces bactériennes, les domaines ou motifs conservés des produits de gène. Ces informations ont permis de faire ressortir une implication positive ou négative potentielle dans l'établissement d'une infection et de concentrer nos efforts sur quelques mutants sélectionnés.

Mutagénèse génomique en masse et STM

La STM (Signature-Tagged Mutagenesis) est une technique permettant de rechercher des facteurs de virulence à grande échelle, par sélection négative *in vivo,* en construisant des mutants par transposition (Shea *et al*., 2000). Chaque mutant à tester contient sa signature d'ADN personnelle via un oligonucléotide qui le rend unique. À l'origine, la STM a été créée utilisant un système de criblage à hybridation différentielle (Hensel *et al*., 1995), mais nous avons raffiné la technique en criblant par PCR, ce qui donne des résultats plus spécifiques tout en étant plus sensible (Lehoux *et al*., 2002a; Lehoux *et al*., 2002b). L'absence d'un mutant du groupe de récupération à la sortie du modèle animal comparativement à sa présence dans le groupe d'entrée constitue un mutant atténué (voir la figure 6 et l'article en appendice 1 de la présente thèse). Nous avons choisi d'utiliser la STM à d'autres techniques comme IVET (In Vivo Expression Technology) (Chiang *et al*., 1999) car la STM est une méthode relativement rapide, permettant de chercher des gènes de virulence à l'intérieur d'une population mixte de mutants utilisant le même animal.

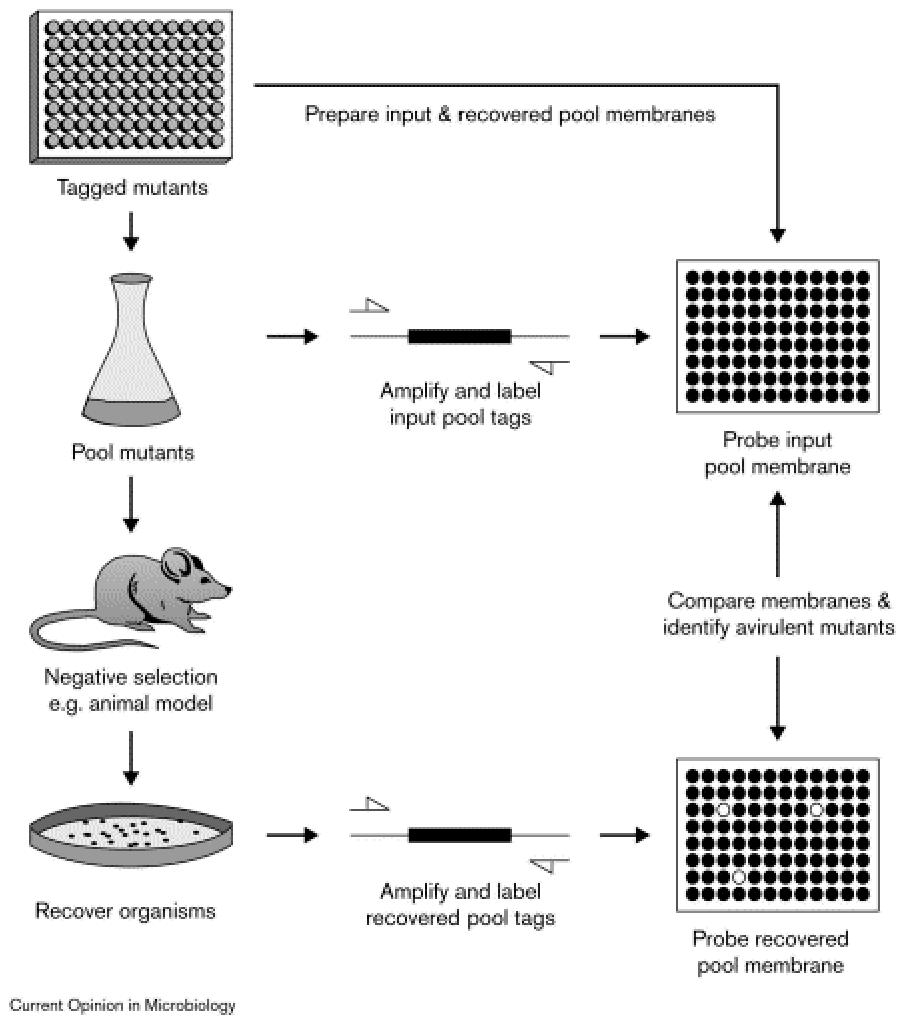

Figure 6. Schéma de l'approche globale de mutagenèse en masse «Signature-Tagged Mutagenesis» (STM), sélection négative *in vivo* suite au passage dans un modèle animal approprié et criblage par PCR. (Figure tirée de Shea *et al*., 2000)

Le modèle animal

Des modèles animaux naturels de la FK n'ont pas encore été identifiés à ce jour, et ce malgré un effort soutenu de criblage chez les primates non humains (Glavac *et al*., 2000) résultant en une panoplie de modèles animaux développés. L'administration d'une suspension libre de *P. aeruginosa* dans les voies respiratoires d'un mammifère, comme le rat ou la souris, constitue un excellent modèle d'étude des infections aigues à *P. aeruginosa*. Cependant, comme *P. aeruginosa* est un pathogène opportuniste, la dose est cruciale ; si l'inoculum est trop faible, il y aura exclusion par l'hôte et si l'inoculum est plus élevé, l'organisme mourra d'une septicémie aigue (Southern *et al*., 1970). Cash et collaborateurs (1979) ont développé un modèle d'étude qui est rapidement devenu le modèle de prédilection dans l'étude des infections pulmonaires chroniques à *P. aeruginosa* : le modèle des billes d'agar chez le rat. Il est maintenant bien établi que le fait d'inclure l'inoculum bactérien à l'intérieur de billes d'agar, a pour effet de protéger celui-ci des défenses de l'hôte et de l'exclusion mimant ainsi l'épaisse couche d'alginate protectrice. De plus, les dommages tissulaires causés par l'inflammation dans les poumons FK sont sensiblement les mêmes que l'on retrouve dans un contexte d'infection pulmonaire chronique chez le rat (Cash *et* al., 1979). Brièvement, un inoculum standardisé à 1×10^6 cfu total de cellules de *P. aeruginosa*, provenant d'une culture de seize heures, est mélangé à une solution d'agarose maintenue liquide à 55°C. Par la suite, la suspension de cellules et d'agarose est ajoutée graduellement à de l'huile minérale en agitation rapide, également à 55°C, dans le but de former une émulsion. Finalement, l'émulsion est rapidement refroidie en ajoutant de la glace à l'extérieur du contenant et les billes sont récoltées et lavées par décantation utilisant un sel biliaire, le déoxycholate de sodium. La taille moyenne des billes ainsi générées est de l'ordre de 10 à 100 µm (voir la figure 7).

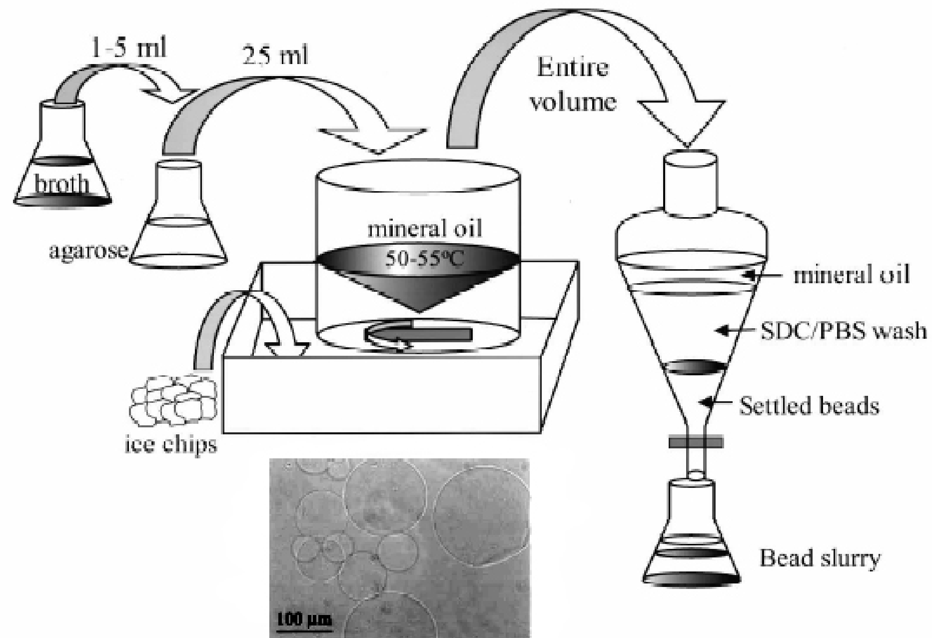

Figure 7. Méthode d'inclusion et de préparation des cellules de *P. aeruginosa* dans les billes d'agar qui serviront d'inoculum pour l'infection pulmonaire chronique chez le rat. (Figure tirée de Van Heeckeren and Schluchter, 2002)

Chapitre 2

Régulation transcriptionnelle chez *Pseudomonas aeruginosa*

Le chapitre 1 constitue un article de revue soumis pour évaluation à la revue *Molecular Microbiology*. Cet article regroupe les principales données connues ainsi que les récentes découvertes concernant la régulation transcriptionnelle de *Pseudomonas aeruginosa* basée sur les facteurs sigma en mettant l'emphase sur les facteurs sigma de type ECF (extracytoplasmic functions). La rédaction de ce manuscrit a été réalisée par moi-même selon une idée originale et de judicieux conseils du Dr Levesque.

Résumé

Chez *Escherichia coli*, la transcription est dépendante de 7 facteurs sigma : Un facteur sigma-70 essentiel, σ^{70}, ainsi que de six facteurs alternatifs dispensables, σ^{28}, σ^{32}, σ^{38}, σ^{54}, σ^{E} et σ^{FecI}. La transcription des gènes de *E. coli* est modulée par la présence de plus de 350 facteurs transcriptionnels de différentes familles souvent décrites pour la première fois chez cette bactérie. Par contre, l'analyse du chromosome de *P. aeruginosa* a révélé la présence de 24 facteurs sigma parmi les 5570 cadres de lecture ouverts du génome. *P. aeruginosa* possède aussi un facteur sigma essentiel très homologue à σ^{70} de *E. coli* (*rpoD*) ainsi qu'un seul facteur sigma-54 (*rpoN*) aussi très homologue à σ^{54} de *E. coli*. *P. aeruginosa* possède également un homologue de σ^{32}, *rpoH*, impliqué entre autres dans la réponse au choc thermique. Aussi présents sur le chromosome de *P. aeruginosa*, *rpoS*, un homologue de σ^{38} impliqué dans la communication cellule-cellule au début de la phase stationnaire de croissance. Tous les autres facteurs sigma de *P. aeruginosa* sont de la sous-famille des ECF et regroupe *fpvI*, *pvdS*, *algU*, *sigX* et 15 autres inconnus.

Summary

In eubacteria, the expression of genes is tightly controlled by many transcriptional regulators. Basically, the primary description of all these regulators originated from the well-known bacterium *Escherichia coli*. The arsenal of transcriptional regulatory proteins of *E. coli* includes 7 sigma factors which are involved in core RNA polymerase interaction and promoter recognition. *E. coli* also encodes up to 350 transcriptional regulators from different families to modulate the expression of these genes. Now, five years have passed since the releasing of the genome sequence of the opportunistic pathogen *Pseudomonas aeruginosa* strain PAO1. Information acquired from the whole sequence of PAO1 revealed the presence of 24 putative sigma factors, 19 of which are from the ECF (extracytoplasmic functions) subfamily. This paper reviews all the actual knowledge regarding the sigma factors of *P. aeruginosa* with a particular emphasis on the known and unknown ECF members.

Introduction

Scientific research over the past decade has doubtlessly revealed an awesome quantity of information generated by the genomic effort. To date, the National Center for Biotechnology Information (NCBI) has published 229 complete microbial genomes and 373 genomes in progress since the *Haemophilus influenzae* sequence was completed in 1995 (http://www.ncbi.nlm.nih.gov/genomes/). In addition to the challenge of understanding the function of all genes annotated in a defined genome, a difficult challenge is coming up-understanding global gene regulation. Since the appearance of new global transcript profiling technologies such as Microarrays or, more recently RNAi-based regulation, we now have the potential to make such an attempt. Comparative genomics could presumably reconstruct metabolic networks. However, the analysis of transcriptional regulatory networks are hindered by the low level of evolutionary conservation of transcriptional regulators (Herrgard *et al.*, 2004). An additional hurdle is the distribution of the genes encoding these proteins widely throughout the genome. The opportunistic pathogen *Pseudomonas aeruginosa* strain PAO1, encoding one of the largest eubacterial sequenced genomes (6.3Mb), is one of the prototypical examples of the complex network of regulation controlling gene expression. *P. aeruginosa* is known for its incredible

metabolic diversity and capacity for adaptation. As an opportunistic pathogen, *P. aeruginosa* has a remarkable capacity to infect a large array of life-forms including mammals, insects and plants (Lyczak *et al.*, 2000). The exquisite control of *P. aeruginosa* gene expression relies on a collection of transcriptional regulators, sigma factors and more precisely, those members of the ECF subfamily. This paper presents a compilation of sigma factors and transcriptional regulators involved in the reconnaissance of gene promoters retrieved from the complete sequence of *P. aeruginosa* PAO1 strain.

Bacterial transcription

The basic initiation of eubacterial transcription is performed by the multisubunit RNA polymerase ($\alpha_2\beta\beta'\omega$) that binds to a dissociable sigma (σ^{70}) factor (Helmann and Chamberlin, 1988). The sigma-70 (70 kDa) factors are responsible for promoter recognition at -10 and -35 bp DNA elements and the melting of double stranded DNA when it binds to the core RNA polymerase (Gruber and Gross, 2003). *Escherichia coli* possesses one housekeeping sigma subunit that directs most of the transcription during exponential growth and a pool of 6 alternatives sigma factors implicated in the transcription of specific regulons associated with environmental or physiological changes (σ^{28}, σ^{32}, σ^{38}, σ^{54}, σ^{E} and σ^{FecI}) (Blattner *et al.*, 1997).

On the basis of gene structure and function, members of the sigma-70 family can be divided into four main groups (Paget and Helmann, 2003). The first group consists of the essential primary sigma factors and are closely related to σ^{70} of *E. coli*. The second group include members also closely related to σ^{70} but which are usually dispensable. The proteins from the third group are more distantly homologous to σ^{70} and are known to activate specific regulons associated with heat-shock or morphological developmental stages such as bacterial sporulation or flagella biogenesis. The fourth group encompasses the largest family and includes the highly divergent extracytoplasmic function factors (ECF) subfamily. This fourth group includes proteins which respond to signals from the extracytoplasmic environment, including detection of misfolded periplasmic proteins (Wosten, 1998).

E. coli has an additional sigma factor, σ^{54} (54 kDa) which is the only sigma factor that is not homologous to σ^{70} (Merrick, 1993). Although most eubacteria encode multiple proteins of the sigma-70 family, they usually have no more than one representative of the sigma-54 family (Gruber and Gross, 2003). Transcription from σ^{54} has distinctive characteristics such as the obligatory presence of a cognate ATP-dependent transcriptional activator which interacts with the sigma-54 subunit and a specific DNA sequence (Buck et al., 2000). Hence, transcription from sigma-54 promoters can be entirely turned-off in the absence of the transcriptional activator. σ^{54}-like proteins are widespread among bacteria and are required for many functions such as nitrogen assimilation, phage-shock response and zinc tolerance (Reitzer, 2003). On another level of complexity, *E. coli* has more than 350 transcriptional regulators that can modulate gene expression to severely control levels of transcripts. These regulators are regrouped in several families based on sequence similarities and information available in literature and include LuxR/UhpA, OmpR, GalR/LacI, LysR, AraC/XylS, ArsR and CRP families (Perez-Rueda and Collado-Vides, 2000).

Pseudomonas aeruginosa PAO1 gene regulation

The regulation of the complex *P. aeruginosa* genome (5570 ORFs) is presumably orchestrated by a high number of transcriptional regulators and two-component regulatory systems as identified by bioinformatics analysis and genome annotation. In theory, these regulatory elements could explain why this bacterium is retrieved in a wide range of environmental niches. When compared with other bacterial systems, the most striking over-representations in *P. aeruginosa* are LysR, AraC, ECF sigma factors and two-component regulatory families (Stover *et al.*, 2000). Two-component systems are usually associated with response to environmental changes as well as regulation of expression of virulence traits. Two-component systems are often composed of a sensor kinase and a response regulator which is phosphorylated by the sensor kinase via a transducing signal following an environmental stimulus. The phosphorylated regulator activates the expression of necessary genes for the appropriate response. A recent review described the genetics and biochemistry of these systems. The repertoire of sensor kinases annotated in the PAO1 genome regroups 63 histidine kinases and 64 putative response regulators. A total of 16

atypical kinases, among them 11 with an HPt (Histidine-phosphotransferase) domain and three with independent HPt module have been identified (Rodrigue *et al.*, 2000). The HPt domain, found in many signal transduction proteins, functions as a mediator of the His-Asp phosphorylation (Yaku *et al.*, 1997). These two-component systems based regulation suggests that *P. aeruginosa* encodes complex control strategies with which to respond to environmental challenges. Regulation of the *P. aeruginosa* genome is also characterized by the presence of approximately 120 LysR-type regulators most of which have unknown functions (http://www.pseudomonas.com). For instance, PtxR and MvaT, two LysR-type regulators, were recently shown to modulate the expression of *toxA*, the exotoxin A coding gene (Carty *et al.*, 2003; Westfall *et al.*, 2004). Fifty-nine putative transcriptional regulators are also predicted to possess an AraC/XylS family signature with genes distributed all over into the PAO1 chromosome, including ArgR elements, the arginine-responsive regulator that controls arginine uptake and metabolism (Gallegos *et al.*, 1997; Lu *et al.*, 2004). The other families of regulatory proteins are the LuxR-type including, GacA, LasR and RhlR and GntR, IclR, TetR-type families.

Sigma factor σ^{54} (or RpoN) is the unique sigma-54 like transcriptional regulator protein found in the *P. aeruginosa* chromosome to date. To proceed with initiation of transcription, the RNAP-RpoN complex must participate in an interaction with a transcriptional activator, involving nucleotide hydrolysis. The Pseudomonas databases predicted 22 proteins having the sigma-54 ATP binding region. Among them, FleQ and FleR are involved in the biosynthesis of flagellin, the major component of the single flagella of *P. aeruginosa* (Dasgupta *et al.*, 2003), PilR for pilin biosynthesis (Mattick *et al.*, 1996) and AlgB that regulates alginate exopolysaccharide production (Ma *et al.*, 1998).

Pseudomonas aeruginosa sigma factors

RpoD (σ^{70})

P. aeruginosa like other gram-negative bacteria has a major sigma-70 factor RpoD that recognizes a large number of promoters controlling expression of housekeeping genes. The RpoD protein has extensive homology with the principal *Escherichia coli* σ^{70}, indicating that the σ^{70} has an identical function. It was shown that the *P. aeruginosa* σ^{70} can complement a temperature sensitive mutation of the *E. coli rpoD* gene (Tanaka and Takahashi, 1991). RpoD dependent transcription is essentially performed during the exponential phase of growth (Fujita *et al.*, 1994). The *rpoD* gene is transcribed from two promoters, P_C and P_{HS}. Synthesis of *rpoD* mRNA from P_C is constitutive under both steady-state and heat-shock growth conditions, while that of P_{HS} is transiently induced upon heat-shock (Aramaki and Fujita, 1999). The promoter consensus sequences recognized by RpoD in the -35/-10 region of the transcriptional initiation site are highly similar to those from *E. coli* σ^{70}. Hence, the *E. coli* -10 element (**TATAAT**) and the *P. aeruginosa* (**T**AtAA**T**) are highly similar; whereas the -35 element of *P. aeruginosa* (**TTG**a**C**c) is slightly different from the *E. coli* consensus (**TTGACA**) (bases in bold uppercase letters and plain uppercase letters are present in more than 50% and 40% of the sequences, respectively, whereas bases in lower case letters are present in more than 30% of the sequences over 149 RpoD-dependent promoters aligned together)(Ramos, 2004).

Sequence alignment of σ^{70} like proteins from different genus of eubacteria led to the identification of four highly conserved regions in amino acid composition (Helmann and Chamberlin, 1988). These regions, 1 to 4, have been subdivided further with the accumulation of sequence data (Lonetto *et al.*, 1992). Region 1, the less conserved, is divided into two distinct regions 1.1 and 1.2. Region 1.1 is found only in the primary sigma-70 factors, such as σ^{70} and RpoD, and was shown to be involved in the modulation of DNA binding and in the efficiency of initiation of transcription. Region 1.2 is probably involved in the formation of an open complex. This region is present in all sigma-70 subfamilies except for the ECF subfamily of proteins (Wilson and Dombroski, 1997). Region 2, the most conserved, is divided into 4 subregions 2.1, 2.3, 2.4 and 2.5. Region 2.1 is involved in core RNAP recognition, region 2.3 in melting of DNA, region 2.4 recognize

the -10 element and region 2.5 recognizes a -14/-15 element in *E. coli* and -16 element in *Bacillus subtilis* (Harley and Reynolds, 1987; Voskuil *et al.*, 1995). Regions 3 and 4 are both divided into two subregions. Region 3.1 contains a helix-turn-helix DNA-binding motif and the less conserved 3.2 region may be involved in binding the RNAP core enzyme (Zhou and Gross, 1992). Region 4.1 binds transcriptional activators during initiation of transcription and region 4.2 recognizes the -35 element (Harley and Reynolds, 1987). Upon comparison of both, *P. aeruginosa* RpoD and *E. coli* σ^{70}, it is noteworthy that regions 2.4 and 4.2, involved in interaction at -10/-35 promoter elements, are 100% identical. The identities found in these regions involved in promoter specificity are in agreement with the cross-recognition of the RpoD-dependent and σ^{70}-dependent promoters assayed to date. Moreover, when necessary, *E. coli* RNAP responds to activation-repression mechanisms provided that the corresponding regulatory gene is also present (Ramos, 2004).

RpoH (σ^{32})

The similarities in gene regulation between *E. coli* and *P. aeruginosa* regarding *rpoD* and σ^{70} can be extrapolated with experimental data to another σ^{70}-like factor, RpoH, which shows 61% identity to the σ^{H} (sigma 32) protein of *E. coli* (Benvenisti *et al.*, 1995). In *E. coli*, σ^{H} is responsible for the heat-shock induction response in the upshift of temperature from 30°C to 42°C. σ^{H} is required for positive regulation of heat-shock genes as well as the basal expression of more than 20 heat shock proteins, which are molecular chaperones, including DnaK, DnaJ, GrpE, GroEL and GroES as well as specific proteases (Arsene *et al.*, 2000). Transcription of *P. aeruginosa rpoH* gene was shown to be dependent on AlgU, an homolog of *E. coli* σ^{E}. The AlgU-dependent promoter of *rpoH* was found to be activated in mucoid *mucA* mutants, suggesting that conversion to mucoidy and the heat-shock response are co-ordinately regulated in *P. aeruginosa* (Schurr and Deretic, 1997). A number of σ^{H} homologs have been cloned from Gram-negative bacteria that belong to the gamma or the alpha subdivisions of proteobacteria; and it has been reported that the expression of these foreign homologues into *E. coli* Δ*rpoH* activated the transcription of DnaK and GroEL from the start sites normally used in *E. coli* by σ^{H} (Nakahigashi *et al.*, 1998). The level of *rpoH* transcription in *P. aeruginosa* cells was found to be very low at 30°C but was markedly elevated upon a temperature shift to 42°C, an observation

previously made with *E. coli*. The increased levels of RpoH upon heat shock treatment resulted from both the increased synthesis and stabilization of the normally unstable RpoH protein (Nakahigashi *et al.*, 1998).

FliA (RpoF) (σ^{28})

Another alternative sigma factor described in several bacterial genera and found in *P. aeruginosa* is the sigma-28 like FliA (RpoF). The *fliA* gene was first identified in *P. aeruginosa* by using an heterologous probe from *Salmonella typhimurium*. Amino acid sequence analysis revealed that both proteins shared up to 67% similarity. The major function associated with FliA in *P. aeruginosa* is the control of flagellin biosynthesis. In fact, the *P. aeruginosa fliA* gene was able to complement the motility defect of an *E. coli fliA* mutant, but only when transcription was driven from the vector promoter. Insertional inactivation of the *fliA* gene with a gentamicin gene cassette rendered *P. aeruginosa* nonmotile; it was unable to express the flagellin gene (Starnbach and Lory, 1992). The 5' region of *fliC*, the structural gene of flagellin, contains potential RpoN-specific promoters as well as a promoter sequence recognized by FliA itself. Analysis of this promoter region as well as transcriptional start site mapping implicated FliA, and not the RpoN consensus sequences as the functional promoter of the flagellin gene (Totten and Lory, 1990). In *P. aeruginosa*, recent studies on the regulation flagellin synthesis identified the *flgM* gene encoding the anti-sigma 28 factor. The role for the *flgM* gene in motility was demonstrated by its inactivation. The beta-galactosidase activity of a transcriptional fusion of the *fliC* promoter to *lacZ* was upregulated in the *flgM* mutant, suggesting that the activity of FliA was increased. Consistent with these results, an increased amount of flagellin was demonstrated in the *flgM* mutant by Western blot, suggesting that FlgM negatively regulates transcription of *fliC* by inhibiting the activity of FliA. Direct interaction of FlgM with the alternative sigma factor FliA was demonstrated by utilizing the yeast two-hybrid system (Frisk *et al.*, 2002). Molecular mechanisms that control the expression of *fliA* remain unknown but transcription appears to be constitutive and independent of RpoN or other flagellar regulator such as FleQ or FleR (Dasgupta *et al.*, 2003).

RpoS (σ^{38})

The functions attributed to the RpoS alternative sigma factor in *P. aeruginosa* has been recently compared to its homolog (σ^{s}) in *E. coli* and shown to be quite different (Venturi, 2003). Moreover, recent data concerning the linking of the RpoS regulon with the quorum sensing circuit makes it one of the best characterized alternative sigma-70 factor in *P. aeruginosa* (Schuster *et al.*, 2004; Whiteley *et al.*, 2000). Globally, RpoS has effects at the onset of the stationary phase of growth and is clearly involved in the regulation of quorum sensing via the modulation of the transcriptional regulators RhlR and LasR (Latifi *et al.*, 1996). The secretion of autoinducer molecules implicated in the cell-to-cell signaling system of *P. aeruginosa* also directs the formation of biofilm; all these morphological and physiological changes are RpoS dependent (Davies *et al.*, 1998). RpoS was shown to be involved in secretion of virulence factors such as alginate, exotoxine A, LasA and LasB elastases and exoenzyme S (Hogardt *et al.*, 2004; Sonnleitner *et al.*, 2003; Suh *et al.*, 1999). RpoS is also known to be important for survival under stressful conditions. In fact, stationary-phase cells of RpoS-negative strains were less resistant to the exposure to hydrogen peroxide, high temperature, hyperosmolarity, low pH and ethanol (Cochran *et al.*, 2000; Jorgensen *et al.*, 1999).

RpoN (NtrA) (σ^{54})

In *P. aeruginosa*, RpoN is the only member of the sigma-54 like family that is a typical case in eubacteria. As a general rule for most alternative sigma factors of *P. aeruginosa*, the amino acid sequence of the *rpoN* product from *P. aeruginosa* shares 67% similarity with the sequence of σ^{54} of *E. coli*. Initial observations on RpoN functions were linked solely to nitrogen assimilation. However, the discovery of additional genes transcribed by RNAP complexed with RpoN that are not a necessary part of the nitrogen metabolism pathway suggest novel functions for RpoN (Gussin *et al.*, 1986). In fact, these functions have been identified as motility, transport of nutrients, formation of pili, mucoidy and cell-to-cell signalling (Boucher *et al.*, 2000; Dasgupta *et al.*, 2003; Heurlier *et al.*, 2003; Ishimoto and Lory, 1989; Mattick *et al.*, 1996; Strom and Lory, 1993; Thompson *et al.*, 2003; Totten *et al.*, 1990). By modulating different virulence determinants, RpoN has been strongly linked to the virulence of *P. aeruginosa* as well as to its ability to efficiently colonize several hosts

including mammals, insects, nematodes and plants (Hendrickson *et al.*, 2001). All these RpoN-regulated genes have a consensus promoter sequence of -24(GG)/-12(GC); their expression required at least one transcriptional activator (Thony and Hennecke, 1989). The formation of pili and flagella in *P. aeruginosa* is under the control of RpoN. All *rpoN* mutants showed significant reduction of adherence to epithelial cells and tracheobronchial mucin (Chi *et al.*, 1991; Ramphal *et al.*, 1991; Simpson *et al.*, 1992). RpoN mutants have been used as controls defective in adherence in many adhesion experiments and infection models (Comolli *et al.*, 1999). Moreover, RpoN negatively regulates the expression of *sadB*, an essential gene for surface adhesion (Caiazza and O'Toole, 2004). The interaction of RpoN with PilR, a member of a two-component transcriptional regulatory system, was shown to control the expression of type 4 fimbriae (Mattick *et al.*, 1996). The presence of *pilRS* and *rpoN* genes is required for the expression of *pilA*, the structural gene for type IV pilin, whereas any of these are essential for *pilBCD* expression (Boyd and Lory, 1996; Koga *et al.*, 1993). Flagella, as well as type IV pili, are also important structures involved in attachment and RpoN was shown to control flagella-based adhesion and motility (Totten *et al.*, 1990). Studies indicate that both motility and adhesion are regulated by a two-component regulatory system called *fleRS*, which in turn is controlled by another regulator in a cascade that involves *rpoN*. A *fleR* mutant possessing pili adheres poorly to mucins, confirming that a flagellar protein and not pili play a major role in adhesion to mucin (Ramphal *et al.*, 1996). Another transcriptional regulator, *fleQ*, also regulates mucin adhesion and motility in *P. aeruginosa*. Promoter fusions demonstrated that the expression of *fleRS* was dependent on RpoN but the expression of *fleQ* was RpoN-independent (Arora *et al.*, 1997). RpoN coupled with the action of the transcriptional activator FleQ is involved in the expression of *fliD*, the flagellar cap protein involved in mucin adhesion (Arora *et al.*, 1998; Jyot *et al.*, 2002). RpoN obviously controls the quorum sensing system via a positive regulation of RhlI, the auto-inducer synthase responsible for the synthesis of N-butyryl-L-homoserine lactone (Thompson *et al.*, 2003). Moreover, in *rpoN* mutants, it was shown that the expression of the *lasR* and *lasI* genes was elevated at low cell densities, whereas expression of the *rhlR* and *rhlI* genes was markedly enhanced throughout growth (Heurlier *et al.*, 2003). The RpoN alternative sigma factor is also implicated in the regulation of transporters. Deficiency in RpoN abolished the expression of *oprE* encoding a channel-

forming outer membrane protein under aerobic conditions, but did not affect the expression under anaerobic conditions. One mutation on the putative RpoN recognition site also caused reduction of *oprE* expression. The regulation of *oprE* transcription is directly or indirectly controlled by RpoN but also requires some other regulatory proteins bound to the upstream region (Yamano *et al.*, 1998). Hence, the sigma-54 like alternative sigma factor RpoN is implicated at different stages for the regulation of *P. aeruginosa* virulence factors. Mutations in *rpoN* in all cases leads to a reduction in virulence; this is visualized as defects in adhesion, motility and cell-to-cell communication observed in several models of infection.

ECF sigma factors

In 2002, JD Helmann published an exhaustive paper on the extracytoplasmic function sigma factors (Helmann, 2002), regrouping the knowledge on the different ECF proteins across most bacterial genera. In 1994, Lonetto *et al.*, described for the first time a novel subgroup of 8 proteins that showed similarity in amino acid sequences including *Myxococcus xanthus* CarQ, *P. aeruginosa* AlgU, *Pseudomonas syringae* HrpL, *E. coli* sigma E, *Alcaligenes eutrophus* CnrH, *E. coli* FecI, *Bacillus subtilis* SigX and *Streptomyces coelicolor* sigma E (Lonetto *et al.*, 1994). As mentioned above, the ECF subfamily is a branch of the sigma-70 like sigma factors. ECF are small regulatory proteins that are quite divergent in sequence when compared to other sigma factors. At least three common characteristics are shared among all ECF members. The first one is they often recognize promoter elements with an (AAC) motif in the -35 region. In many cases the ECF sigma factor is co-transcribed with a transmembrane anti-sigma factor having an extracytoplasmic sensory domain and an intracellular inhibitory domain. Finally and most important, they are mainly associated with extracellular functions. Among these functions, we find regulation of periplasmic stress and heat-shock (σ^{E}), iron transport (σ^{FecI}), metal ion efflux system (CnrH), alginate secretion (AlgU) and synthesis of membrane-localized carotenoids (CarQ). The analysis of completed microbial genomes revealed a correlation between genome size and the number of predicted genes encoding sigma factors. In fact, the model actinomycete *S. coelicolor* A3(2) (8.7 Mb) encodes an incredible 65 putative sigma factors and at least 45 proteins of the ECF subfamily; these proteins are potentially responsible for

a response to disulphide stress, cell wall homeostasis and aerial mycelium development (Bentley *et al.*, 2002). The 6.3 Mb *P. aeruginosa* genome encodes 24 sigma factors, among which, 19 are from the ECF subfamily. Only three have a characterized function: AlgU is involved in alginate biosynthesis (Schurr *et al.*, 1996), PvdS is involved in iron regulation responsible for biosynthesis of pyoverdine (Cunliffe *et al.*, 1995) and SigX was shown to control the expression of the major outer membrane protein OprF (Brinkman *et al.*, 1999).

AlgU (RpoE) (σ^{22})

AlgU shares a striking 79% sequence similarity with *E. coli* σ^E, a heat-shock protein produced in response to growth at elevated temperatures. In *P. aeruginosa*, AlgU has been primarily identified for its importance in conversion from the non-mucoid to the mucoid phenotypes (Martin *et al.*, 1993a; Schurr *et al.*, 1996). It was also shown to be involved in resistance to oxidative and heat shock stress (Martin *et al.*, 1994; Schurr and Deretic, 1997). AlgU can interact with a DNA region upstream of the *algD* gene, the key regulatory enzyme in the alginate biosynthesis pathway that leads to the conversion to mucoidy (Martin *et al.*, 1993a). Circumstantial information has accumulated by which non-mucoid strains convert into mucoid strains and are associated with the establishment of chronic lung infections in CF patients (Govan and Deretic, 1996). Four other genes, *mucABCD* are involved in regulation, (Boucher *et al.*, 1996; Schurr *et al.*, 1996). MucA was shown to act as an anti-sigma factor by binding to AlgU and inhibiting its activity. MucB, another negative regulator of AlgU, was localized in the periplasm. MucB exerts its function from this compartment, since deletion of the leader peptide and the cytoplasmic location of MucB abrogated its ability to inhibit mucoidy (Schurr *et al.*, 1996). It has been demonstrated that *mucA* plays a critical role in the process of mucoidy conversion by (i) the presence of frameshift mutations disrupting the *mucA* coding region in mucoid cells that were absent in non-mucoid parental strains, (ii) genetic complementation of *mucA* mutations with the *mucA+* gene, (iii) allelic exchanges with specific mutant *mucA* genes causing conversion to mucoidy in previously non-mucoid cells, and (iv) detection of identical and additional *mucA* mutations in clinical mucoid strains isolated from the lungs of CF patients. This suggested that the inactivation of *mucA* results in constitutive expression of the alginate pathway dependent on *algU* transcription, and that such mutants

may be selected *in vivo* during chronic infections in CF (Martin *et al.*, 1993a). It was also suggested that mucoid conversion is a response to oxygen radical exposure and that this response is a mechanism of defence by the bacteria. Mathee *et al*. reported that polymorphonuclear leukocytes and their oxygen radicals can cause the phenotypic and genotypic changes which are typical of the intractable form of *P. aeruginosa* found in the CF lung (Mathee *et al.*, 1999). MucB is also known to be a negative regulator of AlgU function. The loss of *mucB* function is sufficient to cause conversion of *P. aeruginosa* to the mucoid phenotype (Martin *et al.*, 1993b). Recently, the screening of a large *P. aeruginosa* mutant library constructed by the Signature-Tagged Mutagenesis (STM) approach in the rat lung showed that *algU* is essential *in vivo* for maintenance of *P. aeruginosa* in the rat lung (Potvin *et al.*, 2003). In fact, the conversion to the mucoid phenotype is associated with the maintenance of a long term lung infection in CF patients (Ramsey and Wozniak, 2005). The establishment of these chronic infections in CF lungs was also shown to be mediated by the repression of the type III secretion system via the upregulation of *algU* in a *mucA* mutant background (Wu *et al.*, 2004). An exhaustive study from Firoved *et al*. using transcriptome profiling focused on the analysis of AlgU dependent promoters (GAACTTN$_{16\text{-}17}$TCcaA) by an *in silico* genomics approach identified 35 new genes regulated by AlgU (sigmulon). They suggested the existence of a previously unknown connection between conversion to mucoidy and the expression of lipoproteins with potential pro-inflammatory activity. This link may be of significance for infections and inflammatory processes in CF (Firoved *et al.*, 2002). Recently, Firoved and Deretic applied microarray analysis on a *mucA* mutant to identify on whole-genome scale genes that were coinduced with the AlgU sigmulon upon conversion to mucoidy. Transcriptome analysis revealed co-induction of a specific subset of known virulence factors including the *lasB* elastase gene, the alkaline metalloproteinase gene *aprA*, and the protease secretion factor genes *aprE* and *aprF* as well as toxic factor (cyanide synthase) (Firoved and Deretic, 2003).

PvdS

PvdS is probably the second most characterized sigma-70 ECF subfamily factor of *P. aeruginosa*. A number of common characteristics from ECF sigma factors have been

shown to be shared by PvdS (Helmann, 2002; Wilson and Lamont, 2000). Nucleotide sequence analysis revealed that *pvdS* shows considerable similarity to σ^{FecI} of *E. coli*, a positive regulator for transcription of the *fec* (ferric citrate transport system) operon (Miyazaki *et al.*, 1995). Other members of this subgroup include PbrA from *Pseudomonas fluorescens*, PfrI and PupI from *Pseudomonas putida*, and are all controlled by the Fur (ferric uptake regulator) repressor. In all cases they activate transcription of genes for the biosynthesis or uptake of siderophores (Leoni *et al.*, 2000). The major functions attributed to PvdS, recently reviewed (Vasil and Ochsner, 1999; Visca *et al.*, 2002), included the regulation of pyoverdine siderophore biosynthesis (Cunliffe *et al.*, 1995), exotoxine A (Ochsner *et al.*, 1996) and the endoprotease PrpL (Wilderman *et al.*, 2001). When grown under iron-deficient conditions, *P. aeruginosa* produces pyoverdine, a fluorescent yellow-green siderophore of low molecular weight. The *pvdS* gene is required for the expression of these pyoverdine synthesis genes. The *pvdS* gene is expressed only in iron-starved bacteria and is regulated via the Fur repressor protein (Cunliffe *et al.*, 1995). Fur is a well known and crystallized holorepressor that interacts with the DNA operator sequences (fur box) to tightly control the expression of the iron-regulated subset genes (Ochsner *et al.*, 1995). It was shown that in the presence of a sufficient amount of iron, Fur is able to repress the transcription of *pvdS* and consequently repressed the expression of *pvdA*, which encodes a key enzyme of the pyoverdine biosynthetic pathway (Leoni *et al.*, 1996). The production of exotoxine A is also secreted in iron-limiting conditions. The exotoxine A structural gene, *toxA*, is regulated at the transcriptional level by the gene products of *regAB*. The expression of both *toxA* and *regAB* is repressed under iron-deficient conditions, suggesting a role for Fur in regulation of *toxA* expression. It was shown that the control of exotoxine A, which is related to iron concentration, is mediated by PvdS *in vitro* and in lung infections associated with CF (Ochsner *et al.*, 2002). In a *pvdS* deletion mutant, exotoxine A was produced at low levels of less than 5% compared to wild-type, but still in response to iron starvation (Ochsner *et al.*, 1996). These observations suggested that another regulatory mechanism, in addition to the Fur-PvdS system, was involved in iron regulation of exotoxine A production. This regulator is PtxR, a LysR type transcriptional regulator, which was shown to positively affect the transcription of *toxA* and *regA* (Hamood *et al.*, 1996). Iron-regulated transcription of *ptxR* was demonstrated from a P2 promoter under microaerobic conditions,

but not constitutive expression from another promoter called P1, was dependent on *pvdS*, even under aerobic conditions (Vasil *et al.*, 1998). PtxR and PvdS were also shown to be implicated in the synthesis of pyoverdine via a positive regulation of the *pvdABCD* operon which is negatively regulated by high-iron conditions (Stintzi *et al.*, 1999). Recently, the analysis of iron starvation response by the GeneChip technology lead to the identification of novel genes involved in the biosynthesis of pyoverdine (Ochsner *et al.*, 2002). Under low-iron conditions, expression of 26 genes or operons was reduced in a *pvdS* mutant compared with wild type, including numerous novel pyoverdine biosynthetic genes (Ochsner *et al.*, 2002). It is known that pyoverdine is able to chelate extracellular iron and the resulting pyoverdine iron-complexe is transported back into the cell via the FpvA transporter. The binding of iron-free pyoverdine to FpvA transduces a signal to the periplasmic part of the membrane-spanning anti-sigma factor FpvR. The signal is transmitted to the cytoplasmic part of FpvR, which controls the activity of PvdS (Lamont *et al.*, 2002; Poole *et al.*, 1993). It was shown that FpvR negatively regulates the activity of a second ECF sigma factor, FpvI, which is required for the synthesis of FpvA, and the presence of pyoverdine complexed to iron significantly increases the activity of FpvI so that production of FpvA is induced. This is the only example of a branched signalling system of this sort and the first example of an anti-sigma factor protein (FpvR) that directly regulates the activities of two different ECF sigma factor proteins (PvdS and FpvI) (Beare *et al.*, 2003).

SigX

SigX is the last sigma-70 factor of the ECF subfamily with an attributed function that has been demonstrated experimentally in *P. aeruginosa*. SigX shares 49% similarity to sigma-W of *Bacillus subtilis* which is induced by different stresses such as alkaline shock, salt shock, phage infection and certain antibiotics that affect cell wall biosynthesis (Schobel *et al.*, 2004). The gene encoding OprF, a major outer membrane protein in *Pseudomonas* sp., was previously thought to be constitutively transcribed from a single RpoD promoter immediately upstream of the gene. In *P. aeruginosa* as well as in *P. fluorescens*, the *sigX* gene is located immediately upstream of *oprF* and the disruption of *sigX* in both species significantly reduces *oprF* expression (Brinkman *et al.*, 1999).

Unknown Sigma Factors

Of the 24 putative and known sigma factors found in the entire PAO1 genome sequence, 15 have no predicted functions except for the common theme of belonging to the ECF subfamily (Table 2). These proteins were found using a keyword search with the Pseudomonas.com website along with the structural feature PS01063 from Prosite. These 15 putative sigma-70 factors, presumably belonging to the ECF subfamily were sorted according to their PA number. In Table 2, we indicated relevant characteristics including the predicted protein molecular mass, as calculated using the COMPUTE PI/MW program from the ExPASy Proteomics Server (www.expasy.org). We also included in Table 2, characterized and uncharacterized homologous proteins from other bacterial genera, and those found in *P. aeruginosa*. Homology is expressed as a similarity value (in percentage) including positives and identical residues found by using the BLASTP program from National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). As mentioned in the literature, ECF sigma factors are often co-transcribed with a putative, inner-membrane anchored, sensor that could act as a signal transducer and a negative regulator (anti-sigma) (Helmann, 2002). The right portion of Table 2 regroups the main characteristics of these co-transcribed genes that we identify here as anti-sigma factors. PA numbers, molecular mass and homologues were identified and described. Moreover, we added an important set of data concerning putative positions of the transmembrane helix. Using the DAS program (www.sbc.su.se/~miklos/DAS), that measures hydrophobic potential, we significantly predicted in most cases at least one transmembrane segment. These putative transmembrane helices were confirmed by running topology scans with other known programs from the Expasy Proteomic Server.

The most striking feature of these unknown proteins is the high homology values shared with ECF sigma factors involved in regulation and uptake of ferric citrate (Visca *et al.*, 2002). The ferric citrate transport system of *E. coli* involves three specific proteins: FecR, the inner-membrane sensor that transduces signals to FecI, the sigma factor, ECF subfamily that bind to core RNAP in the cytoplasm. FecI directs transcription from the promoter upstream of the *fecABCDE* transport genes. FecA, an outer-membrane protein, was also shown to be essential in the signal cascade (Braun and Braun, 2002). Moreover, transcription of the *fecIR* regulatory genes and the *fec* transport genes is repressed by the

Fur protein when loaded with iron. Two *fecIRA* systems have been reported to date in *P. aeruginosa*, the *fiuIRA* (PA0470-0472) and *fpvIRA*. FiuA is an outer membrane receptor protein responsible for the transport of the ferrioxamine, a probable hydroxamate-type ferrisiderophore. FiuA is under the transcriptional control of FiuIR also responsible for signal transduction and transcriptional response to a ferridoxamine cue (Vasil and Ochsner, 1999). As described by Visca et al., 11 of the 15 unknown ECF sigma factors presented in Table 2, excluding PA1351, PA2093, PA2896 and PA3285, possess a significant match with a fur-binding sequence (GATAATGATAATCATTATC) (Visca *et al.*, 2002). Moreover, many of these were experimentally confirmed to be regulated by the Fur repressor (Ochsner and Vasil, 1996). Homologies presented in Table 2 omit highly significant values from other reported systems linked to the metabolism of siderophores such as PupIR from *Pseudomonas putida* and FpvIR from *P. aeruginosa*. PupIR regulates the biosynthesis and metabolism of the Pseudobactin BN8 via PupB, which is the pseudobactin receptor. PupI and PupR display significant similarity to the FecI and FecR proteins of *E. coli* (Koster *et al.*, 1994).

Transcriptome analysis associated with iron starvation revealed that 6 sigma-70 factors of the ECF subfamily were found to be highly regulated by iron starvation as well as their cognate putative transmembrane sensor (Ochsner *et al.*, 2002). These are PA0471-0472, PA1300-1301, PA2467-2468, PA3409-3410, PA3899-3900 and PA4895-4896. Interestingly, none of them were shown to be differentially regulated by PvdS (Ochsner *et al.*, 2002). Another recent study on the iron-starvation response published microarray results according to the iron-response specific to the exponential growth phase, whereas the previously reported work analysed the response during the stationary phase of growth (Palma *et al.*, 2003). Similar results were obtained in both experiments concerning the unknown sigma-70 factors. Briefly, PA0471-0472, PA2467-2468 and PA3899-3900 were upregulated, while sigma-70 factors PA3410 and PA4896 only (not their cognate sensor). New genes identified as upregulated in this study were PA1363, PA1911 and PA2387 (Palma *et al.*, 2003).

By regulating iron influx and export, as well as other cell envelope regulons such as the biosynthesis of alginate and exoproteases, ECF sigma-70 factor-based regulation plays a

determining role in successful *in vivo* implantation (Bashyam and Hasnain, 2004). In order to associate some putative functions to the unknown sigma-70 factors, ECF subfamily of *P. aeruginosa*, many transcriptome experiments were done using growth in different culture conditions. Mashburn *et al*., published a study where in *P. aeruginosa* was shown to utilize *Staphylococcus aureus* cells as an iron source when co-cultured together in the rat peritoneum (Mashburn *et al*., 2005). In these experiments, they confirmed the implication of 6 sigma/sensors for the acquisition of iron primarily noted by Ochsner *et al*. In fact, all of these operons are induced in the rat peritoneum, an iron-starved environment. The same operons were highly repressed when co-cultured with *S. aureus* cells. PA0149, PA0675 and PA1911-1912 were also shown to be induced *in vivo* but not when co-cultured with *S. aureus* (Mashburn *et al*., 2005). Another study showed that some sigma-70 factors from the ECF subfamily were also important in the interaction with primary normal human airway epithelial (PNHAE) cells (Frisk *et al*., 2004). In fact, PA1300, PA2468, PA3899 and PA4896 were strongly repressed, as well as many other iron-regulated genes, after 12 h of interaction with the PNHAE cells. This suggested that these epithelial cells can serve also as an iron source for *P. aeruginosa* (Frisk *et al*., 2004). It is clear that a subgroup of these sigma-70 factors of unknown function and their cognate sensors (Table 2), not only possess significant homologies to characterized iron uptake regulators, but are highly involved in this kind of regulation.

The recently described VqsR transcriptional regulator of the LuxR family, encoded by PA2591, regulates several virulence factors. Transposon mutation of *vqsR* was shown to abrogate the production of quorum-sensing autoinducers, the secretion of exoproducts and diminished bacterial virulence in the *Caenorhabditis elegans* infection model (Juhas *et al*., 2004). Microarray analysis revealed that 7 ECF sigma factors are upregulated in a *vqsR* mutant including PA0149, PA0472, PA1350, PA1912, PA2468, PA3899 and PA4896, suggesting the linkage of VqsR with the regulation of iron-based virulence traits (Juhas *et al*., 2005). Those indirectly regulated by *vqsR* were also identified to be transcriptionaly promoted by the addition of hydrogen peroxide, except PA1300, PA1350 and PA3410 (Palma *et al*., 2004). It is known that *P. aeruginosa* must often overcome a high concentration of oxidants to successfully infect the human host (Palma *et al*., 2004). This indicated that the response of *P. aeruginosa* to hydrogen peroxide consists of an

upregulation of protective mechanisms such as ECF-type sigma factors coupled to their cognate sensor. Interestingly, from all the unknown sigma factors listed in Table 2, none of them were found to be regulated by the quorum sensing regulon in neither the paper of Schuster *et al.* nor in the paper of Wagner *et al*. (Schuster *et al.*, 2003; Wagner *et al.*, 2004).

An exhaustive study on *P. aeruginosa* genes essential *in vivo* in the chronic rat lung infection model (Potvin *et al.*, 2003) using the signature-tagged mutagenesis approach (STM) based on PCR screening (Lehoux *et al.*, 2004) identified 148 genes attenuated *in vivo*. As previously reported, STM2895 was primarily identified to be defective in the secretion of exoproteases (Potvin *et al.*, 2003). PA2895 in Table 2 is a putative transmembrane sensor of the unknown ECF-type sigma factor. In addition to the implication in extracellular function (exoproteases), PA2895 and PA2896 respond to almost all basic characteristics of ECF sigma factors. PA2896 and PA2895 genes are co-transcribed and PA2895 has a transmembrane helix prediction.

The recent data presented in Table 2 concerning the unknown ECF-type sigma factors of *P. aeruginosa* suggest an implication of several of these sigma factors in the response to iron availability and to hydrogen peroxide. However, the individual role and function of each has to be determined. In his review paper on ECF sigma factors, Helmann suggested different approaches to determine the exact function of an ECF-type transcriptional regulator (Helmann, 2002). First, a mutant strain of the factor under study may have a phenotype, such as PA2895-PA2896, which indicates exoprotease defects, providing indications to a putative function. Second, the identification of the target genes with known functions will suggest a regulation pattern that leads to a visible phenotype. Third, the identification of a specific environmental cue that causes growth defects or any other visible characteristics will also lead to a putative function. These stimuli can be heat, cold, oxidative, metal concentrations, osmolarity, host interaction, nutritional starvation, etc. Many basic techniques from genomics, proteomics and bioinformatics could serve in that kind of investigation.

Conclusion

In conclusion, the 24 sigma factors, more specifically the 19 ECF-type, found distributed all over the genome sequence of *P. aeruginosa* strain PAO1 certainly provide a key explanation for its remarkable abilities of adaptation. These systems work in association by expressing numerous target gene networks to respond to the different cues that will arise in a defined environment or physiological change. We have identified by informatics analysis in the *P. aeruginosa* genome sequence all alternative sigma factors and homologues used by the well-known bacterium *E. coli*. This search includes homologues of the housekeeping sigma factor RpoD, the only sigma-54 RpoN, the stationary phase dependent RpoS, the heat shock RpoH, RpoF for flagella biosynthesis, the pyoverdine siderophore regulator FpvI and RpoE (AlgU) for alginate synthesis and response to heat and oxidative shock. The major difference between these species is that *P. aeruginosa* encodes 17 additional ECF-type sigma-factors in addition to AlgU and FpvI as compared to *E. coli*. The well known Pvds which directs the transcriptional control of pyoverdine biosynthesis as well as exotoxine A, the endoprotease PrpL and SigX which has been recently implicated in transport complete the known sigma factor listing. The 15 remaining unknown ECF sigma factors were analysed *in silico*. Transcriptome data revealed that a majority of the uncharacterized ECF sigma factors were implicated in iron-associated regulation. Even though much of the information still has to be understood, the regulation of the known part of the ECF repertoire, including AlgU, PvdS and FpvI, provides general patterns to the functionality of the regulation-based ECF sigma factors in *P. aeruginosa*.

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Table 1. *Pseudomonas aeruginosa* sigma factors

PA#	Product Name	Gene Name	Function	Putative anti-sigma or transmembrane sensor	Reference
PA0376	sigma factor RpoH	*rpoH*	Heat-shock response		Benvenisti *et al*., 1995; Schurr and Deretic, 1997
PA0576	sigma factor RpoD	*rpoD*	Housekeeping sigma factor		Tanaka and Takahashi, 1991; Aramaki and Fujita, 2000
PA0762	sigma factor AlgU	*algU*	Alginate biosynthesis, Heat-shock, Oxidative stress	mucA, anti-sigma	Martin *et al*., 1993; Wu *et al*., 2004
PA1455	sigma factor FliA	*fliA*	Adhesion, Flagellin biosynthesis	flgM, anti-sigma	Dasgupta *et al*., 2003; Starnbach and Lory, 1992
PA1776	probable sigma-70 factor, ECF subfamily	*sigX*	*oprF* expression		Brinkman *et al*., 1999
PA2387	probable sigma-70 factor, ECF subfamily	*fpvI*	Pyoverdine uptake and regulation	fpvR, anti-sigma	Beare *et al*., 2003; Redly and Poole, 2003
PA2426	sigma factor PvdS	*pvdS*	Iron metabolism, Pyoverdine, Virulence, Oxygen	fpvR, anti-sigma	Cunliffe *et al*., 1995; Beare *et al*., 2003
PA3622	sigma factor RpoS	*rpoS*	Quorum-sensing, Biofilm,		Fujita *et al*., 1994; Hong *et*

			Virulence, Antibiotic resistance	*al*., 2005
PA4462	RNA polymerase sigma-54 factor	*rpoN*	Nitrogen metabolism, Adhesion, Quorum sensing	Ishimoto and Lory, 1989; Hoffmann and Rehm, 2005

Table 2. ***Pseudomonas aeruginosa*** **sigma-70 factors, ECF subfamily with unknown function**

ECF Sigma-70 Factor			**Putative transmembrane sensor/Co-transcribed gene**			
PA# [a]	**MW (kDa)** [b]	**Homologies** [c]	**PA#** [a]	**MW (kDa)** [b]	**TM Helix** [d]	**Homologies** [c]
PA0149	20.3	72% PP4608, 57% PA3410, 52% FecI *E. coli*	PA0150	36.3	1 (99-108)	55 % PP4607, 44% PA3900, 47% FecR *E. coli*
PA0472	19.6	84% PP0352, 57% PA1912, 54% FecI *E. coli*	PA0471	35.4	1 (92-103)	58% PP0351, 47% PA1911, 42% FecR *E. coli*
PA0675	20.6	99% RPA1334, 51% PA1363, 51% FecI *E. coli*	PA0676	35.1	1 (91-104)	55% RPA1335, 43% FpvR *P. aeruginosa*
PA1300	19.8	52% PP4208, 47% PA2468, 47% FecI *E. coli*	PA1301	36.6	2 (92-106) (304-310)	51% PA1364, 43% FpvR *P. aeruginosa*
PA1351	45.6	71% GLL3948, 52% PA0472				
PA1363	27.2	66% RSc2918, 51% PA1300, 45%	PA1364	30.1	1 (45-57)	59% RSc2919, 54% PA1301, 55% FpvR

		PupI *P. putida*				*P. aeruginosa*
PA1912	19.1	75% NE1099, 72% PA3899, 71% FecI *E. coli*	PA1911	35	1 (91-94)	60% NE1098, 56% PA3900, 51% PupR *P. putida*
PA2050	19	69% PA2093, 64% FecI *E. coli*	PA2051	35	1 (90-101)	54% PA2094, 41% PupR *P. putida*
PA2093	19.3	70% PA2050, 66% FecI *E. coli*	PA2094	34.9	1 (88-97)	56% PA2051, 40% RhuR *B. avium*
PA2468	19.9	72% PP0162, 63% PA3899, 63% FecI E. coli, 57% BPP1838, 49% RpoE *B. pseudomallei*,	PA2467	36.4	1 (92-100)	59% PP0161, 55% PA3900, 48% PupR *P. putida*
PA2896	22.3	48% AlgU/SigX *P. aeruginosa*	PA2895	26.9	1 (70-83)	No relevant homologue
PA3285	22.8	52% SigY *X. campestris*, 46% SigX *P. aeruginosa*	PA3286	38.2	No	COG0332 (3-oxoacyl-[acyl-carrier-protein] synthase III)
PA3410	19.8	67% HasI *S. marcescens*, 53% FpvI *P. aeruginosa*	PA3409	35.6	1 (92-99)	56% ECA1539, 51% HasA *S. marcescens*, 46% PA0150

PA3899	19.1	84% PP4611, 75% PupI *P. putida*, 74% PA1912	PA3900	34.9	1 (88-95)	60% BPSS1028, 57% PA2467, 58% PupR *P. putida*
PA4896	20.2	65% BPP2456, 61% PA1912, 53% FecI *E. coli*	PA4895	37.6	1 (104-112)	56% NE1098, 52% PA3900, 50% PupR *P. putida*

a. ORF identification number in the *P. aeruginosa* genome annotated sequence (www.pseudomonas.com)

b. Molecular weight of ORF's amino acid sequences calculated using COMPUTE PI/MW program from the Expasy Proteomics Server (www.expasy.org)

c. Closest homologues found using the BLASTP program from National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Blast hits values are expressed as percentages of similarity. Hits were selected using three features: closest homologue in other genus characterized or not, closest homologue found in *P. aeruginosa* genome and closest characterized homologue. BPP, *Bordetella parapertussis*; BPSS, *Burkholderia pseudomallei*; ECA, *Erwinia carotavora*; GLL, *Gloeobacter violaceus*; NE, *Nitrosomonas europaea*; PA, *Pseudomonas aeruginosa*; PP, *Pseudomonas putida*; RSc, *Ralstonia solanacearum*

Figure legends

Fig. 1. Conserved regions of σ^E from *E. coli* and the related sigma-70 factors from ECF subfamily. Domain 1, which has been implicated in preventing free sigma factors from binding to the promoter, is absent in the ECF subfamily. Domain 2, is the most conserved and contains regions implicated in core binding, DNA melting and interaction with the -10 promoter region. Domain 3 has a very low degree of conservation whereas domain 4 is well conserved containing the helix-turn-helix motif for DNA binding to the -35 promoter region (Figure adapted from Venturi V. published in *Pseudomonas*, volume 2, chapter 12).

Fig. 2. ECF sigma factors are regulated by a common mechanism. In the absence of inducing cues (*left*), the ECF sigma factor is bound by a cognate anti–sigma factor (squiggly line) that is localized to the inner membrane (IM). Inducing cues disrupt the sigma binding activity of the anti–sigma factor, leading to release of the ECF sigma and association with RNA polymerase core enzyme (Figure adapted from Silhavy and Raivio, 2001)

Figure 1

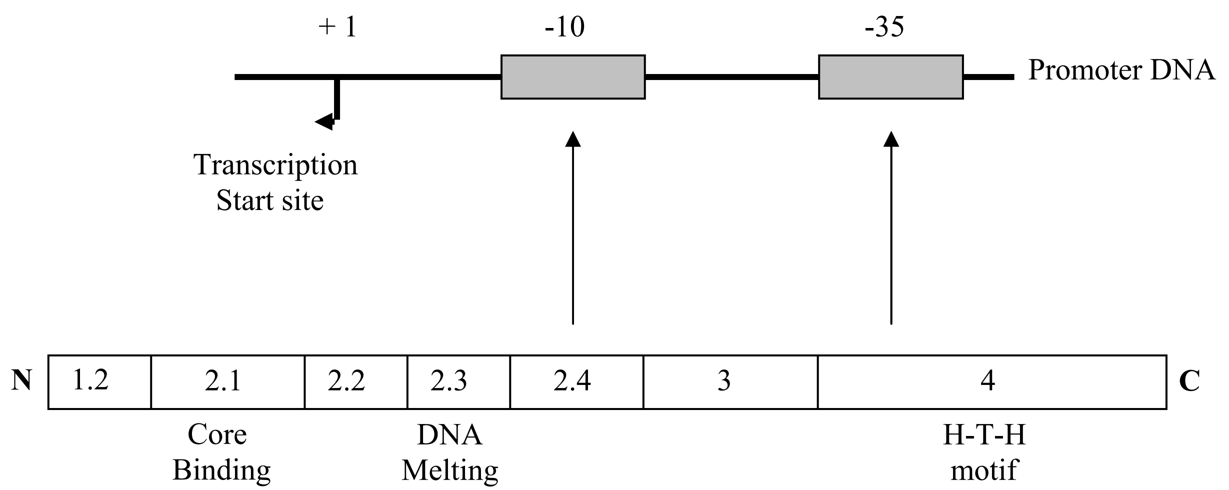
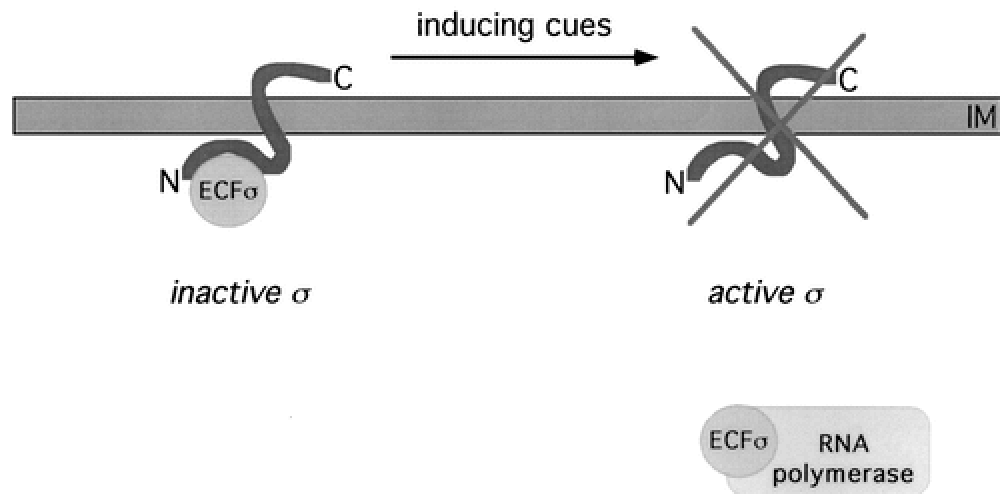


Figure 2



Chapitre 3

Génomique fonctionnelle *in vivo* de *Pseudomonas aeruginosa* et criblage à haut débit de nouveaux facteurs de virulence et de cibles antibactériennes

Ce chapitre présente un article scientifique regroupant la première partie des résultats expérimentaux de mon doctorat publiés dans la revue *Environmental Microbiology* (2003 Dec;5 (12):1294-1308). Dans cet article, nous décrivons le travail qui a été fait sur les 243 mutants STM préalablement identifiés comme défectueux dans le modèle d'infection pulmonaire chronique chez le rat. Les résultats regroupent essentiellement l'identification par séquençage des gènes mutés lors de la transposition et ce, en s'appuyant sur les données du projet de séquençage du génome de *P. aeruginosa* souche PAO1. L'analyse des mutants STM, incluant plusieurs facteurs de virulence connus de la bactérie, nous a permis de valider notre approche expérimentale et ainsi extrapoler sur l'implication des nouveaux gènes identifiés par la même technique. Le reste des travaux porte sur des analyses phénotypiques en regard aux facteurs de virulence connus de *P. aeruginosa*. La construction et le criblage de la banque ont été réalisés par les Drs Sanschagrin et Lehoux. Les clonages qui ont permis l'identification de l'insertion génomique de chacun des mutants ont été faits par moi et Iréna Kukavica. Les analyses phénotypiques et bioinformatiques ont été effectués par moi et Karine Richard. La partie sur la drosophile a été réalisée en collaboration avec le Dr Lau de l'université de Cincinnati. Finalement, la rédaction du manuscrit a été accomplie par les Drs Lévesque, Lehoux et Sanschagrin et moi.

Résumé

Pseudomonas aeruginosa constitue un modèle d'étude clé des pathogènes opportunistes car il est résistant à la plupart des agents antimicrobiens et cause d'importantes infections pulmonaires chroniques. Nous avons développé et adapté la technologie de la STM (signature-tagged mutagenesis) basée sur une réaction multiplexe de polymérisation en chaîne pour effectuer un criblage à haut débit d'une banque de 7968 mutants de *P. aeruginosa* dans le modèle d'infection pulmonaire chronique chez le rat. Après trois rondes de criblage, 214 mutants, représentant des évènements de transposition dans 148 cadres de lecture ouverts, ont été démontrés atténués en virulence dans le poumon du rat et conservés pour analyses subséquentes. Comme preuve à l'appui et accréditant la technologie, nous avons identifié 11 insertions dans des gènes codant pour des facteurs de virulence connus, comme ceux impliqués dans la biosynthèse des pilis, responsables de la motilité et de l'attachement, la synthèse et la régulation de l'alginate, la production d'enzymes extracellulaires telles que l'alkaline protéase, une estérase, une amino peptidase, une transférase de rhamnosyl (bio-surfactant) et une lipopolysaccharide glycosyl-transférase. Des analyses détaillées des 148 mutants STM, incluant 7 auxotrophes, ont révélé des insertions dans 21 des 26 classes de gènes connues des projets de séquençage de génomes bactériens. Nous avons noté qu'au moins 46% des mutants STM possèdent une insertion classée dans les protéines hypothétiques ou de fonction inconnue et qu'environ 40% de tous les mutants STM ont une insertion dans des gènes codant pour des protéines de surface incluant le périplasme et la membrane interne. Étonnamment, 11 mutants STM atténués en virulence ont été également ciblés par des études en transcriptome du quorum sensing et de la mucosité. Le groupe des 130 mutants restants a été testé de façon systématique pour l'expression phénotypique des différents facteurs de virulence connus de la bactérie. De plus, la capacité de ces mutants à infecter le modèle d'étude alternatif *Drosophila melanogaster* a été analysée. Donc, 36 mutants STM ont été montrés défectifs pour la production de protéases, la motilité de type twitching, swimming et swarming, la production de biofilm et la virulence chez la mouche. Finalement, nous avons identifié plusieurs gènes dont l'inactivité réprime fortement la capacité de survie de *P. aeruginosa* dans le modèle d'infection pulmonaire chronique chez le rat.

In vivo functional genomics of *Pseudomonas aeruginosa* for high throughput screening of new virulence factors and antibacterial targets

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Summary

Pseudomonas aeruginosa is a model for studying opportunistic pathogens that are highly resistant to most classes of antibiotics and cause chronic pulmonary infections. We have developed and adapted a multiplex polymerase chain reaction-based signature-tagged mutagenesis (STM) for high throughput screening of a collection of 7968 *P. aeruginosa* mutants in a rat model of chronic respiratory infection. After three rounds of screening, a total of 214 mutants, representing transposition events into 148 open reading frames, were shown to be attenuated in lung infection and were retained for further analysis. As proof of concept supporting this technology, we identified 11 insertions in typical virulence genes such as those coding for pili implicated in motility, attachment and swarming, alginate synthesis and its expression, a mucus transcription regulator, extracellular enzymes such as alkaline protease, esterase and amino peptidase, a rhamnosyl surfactant transferase and a lipopolysaccharide glycosyl transferase. Detailed analysis of the 148 STM mutants, including 7 auxotrophs, revealed insertions in 21 of the 26 known genes classes used to characterize sequenced bacterial genomes. We noted that at least 46% of STM mutants identified had insertions in hypothetical proteins or proteins of unknown function and that ≈40% of all STM mutants had insertions in surface proteins including the outer membrane, the periplasm and the inner membrane. Interestingly, 11 STM mutants attenuated for lung infection were also identified in microarray and transcriptome for quorum sensing and mucoidy production. The remaining 130 mutants were systematically analyzed for their capability to express fully known virulence factors. In addition, testing the ability of these mutants to infect alternative model host *Drosophila melanogaster* revealed 36 STM mutants defective in protease, twitching motility, swimming and swarming. Finally, we identified many genes, the activity of which in respiratory infection was not fully appreciated.

Introduction

The identification of essential bacterial genes *in vivo* not only discovers novel virulence determinants but also indicate which proteins could be used as potential vaccines and antibacterial drug targets. As a model organism for this type of strategy, we are using *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen typically associated with long-term infection in patients such as those undergoing treatment for cancer or burn wounds, trauma and those with cystic fibrosis (Govan and Deretic, 1996). This pathogen has also been identified as the fourth leading cause of nosocomial infection and the major cause of hospital-acquired pneumonia (Jarvis and Martone, 1992; Daniels *et al.,* 2002). Antibiotic therapy of *P. aeruginosa* infections has proved difficult because of the intrinsic resistance of the organism to most families of antimicrobial agents (Hancock, 1988).

Pseudomonas aeruginosa produces a repertoire of virulence factors such as alginate, extracellular proteases including various alkaline proteases, elastases, lipases, a rhamnolipid surfactant and a collection of siderophores including pyoverdin (Meyer *et al.,* 1996; Lamont *et al.,* 2002) In addition to their role in bacterial metabolism and growth, these factors are also implicated in complex virulence phenotypes such as adherence to host cells and tissues, biofilm formation, twitching, swarming and swimming motilities. All the virulence factors presumably make unique and overlapping contributions to bacterial fitness and adaptation during an infection. Understanding the expression of these genes remains the holy grail of research in *P. aeruginosa* infections. However, the complexity of *P. aeruginosa* gene expression *in vivo* during a mammalian infection is very difficult to assess solely by *in vitro* experiments. One may assume then that the *P. aeruginosa* genome must turn on or off genes specific for the host environments encountered *in vivo,* and several discrete cellular steps presumably permit the controlled expression of genes essential for initiation of the infection process and its maintenance. The pattern of *P. aeruginosa* genes expressed solely *in vivo* and essential for infection remains to be investigated if one wants to dissect and have a global vision of the infection versus the pathogen genomic content.

Studies of bacterial genes expressed *in vivo* will be a formidable task requiring several complementary techniques such as *in vivo* expression technology (IVET), signature-tagged

mutagenesis (STM), two-dimensional gel electrophoresis, differential display, chip technology such as microarray and transcriptome profiling, in all cases coupled to systematic bioinformatics analysis (Handfield and Levesque, 1999; Lehoux *et al.,* 1999; Handfield *et al.,* 2000; Whiteley, *et al*., 2001; Choi *et al*., 2002; Firoved and Deretic, 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003). STM is an elegant insertional mutation-based screening method using a population of bacterial mutants for the simultaneous identification of virulence genes important for infection by negative selection (Hensel *et al.,* 1995). This extremely powerful bacterial genetics technique depends upon an *in vivo* selection phenotype in which inactivated genes will diminish bacterial virulence in a model host of infection. One STM screen of *P. aeruginosa* has identified a few novel virulence factors while screening a limited number of mutants using a novel polymerase chain reaction (PCR)-based STM (Lehoux *et al*., 2002). We present our findings of STM on *P. aeruginosa* strain PAO1 using a multiplex PCR-based STM with a 72-tag approach. This work expands significantly on our previous work by screening more than seven times the number of mutants with 7968 mutants using a rat model of chronic respiratory infection. Here, we report the identification of 148 attenuated mutants after multiple rounds of infection in two host models, *Rattus norvegicus* and *Drosophila melanogaster,* as well as systematic analysis of all STM mutants for the expression of known virulence factors. Among the 148 STM mutants, we have identified several attenuated strains in known virulence factors of *P. aeruginosa*, proving that multiplex PCR-based STM is a reliable *in vivo* screening method. In addition, we observed that more than 40% of STM mutants had insertions in genes coding for hypothetical proteins or proteins of unknown function. Importantly, many of these novel mutations affect the expression of key virulence genes such as those involved in biofilm formation, the production of extracellular proteases and the swimming swarming phenotype

Results

Construction of Pseudomonas STM libraries and optimization for in vivo screening

STM was initially used to screen for mutants of *Pseudomonas* that were unable to survive in the rat lung after 7 days of chronic infection (Lehoux *et al*., 2002). In contrast to typical hybridization-based STM, the mutagenesis was done using a miniTn*5*Km2 transposon (DeLorenzo *et al*., 1990) and 11 tags designed for optimal amplification by PCR, which is more sensitive and specific (Lehoux *et al*., 1999). We have now enhanced the capacity of PCR-based STM for high-throughput screening by using multiplexing and 24 specific tags which do not cross-amplify. Instead of synthesizing 72 tags, which could have given problems in PCR co-amplification, we decided to use these 24 tags which were cloned into three distinct miniTn*5*Km, miniTn*5*Tc and miniTn*5*TcGFP in order to facilitate phenotypic selection using antibiotics and green fluorescent protein (GFP) markers. These constructs were used to mobilize 72 STM plasmid tags into *P. aeruginosa* PAO1 by conjugation, and 96 transconjugants were tooth-picked from each mating into 96-well 2 ml microtitre plates. This collection was used to form an *in vitro* input pool containing 72 STM mutants. We reasoned that the utilization of input pools of higher complexity containing 72 STM mutants instead of 11 would diminish the number of animals needed to screen this new collection of 6912 mutants and the collection of 1056 STM mutants constructed previously.

As the complexity of the pools was increased, we sought to increase the number of STM mutants per animal. In preliminary experiments, we tested a series of inoculum sizes: 10^4, 10^5 and 10^6 cfu per animal. Each *in vitro* pool containing either 11 or 72 STM mutants was used to infect animals. Output bacteria were recovered by plating lung homogenates on plates containing appropriate antibiotics, and cfu counts per rat were determined. Chromosomal DNA was prepared from these STM mutants and tested for specific multiplex PCR (see *Experimental procedures* for details). In all cases, we were able reproducibly to amplify specific DNA fragments from each tag by multiplex PCR when the inoculum doses of 10^5 and 10^6 were used. We also noted that some STM mutants that were identified as attenuated and lost in the low-complexity pool were also absent from the high-complexity pool. A collection of 40 strains was chosen randomly and shown by the

Southern transfer analysis to have random insertions of the three miniTn*5*s into the PAO1 genome (data not shown).

Screening of signature- tagged strains and selection of avirulent mutants

To identify *Pseudomonas* genes essential for lung infection and narrow the pool of study, each of the 96 pools containing 72 insertional mutants was used for the assembly of an array of tagged mutants and used to perform three rounds of screening with three rats per array. Each mutant in a defined library has the same tag, but with a high likelihood that it is inserted at a different location in the chromosome from that of other mutants in the same pool. After 7 days of infection, bacteria were isolated from the lungs, and strains missing in the *in vivo* output pool were identified by multiplex PCR. To confirm the identity of each STM mutant, 24 signature tags can be used as a specific primer in combination with a specific antibiotic resistance PCR primer for specific DNA amplifications. Tagged products from arrayed bacterial clone pools can be compared as DNA products of a specific length of 220, 820 and 980 bp are amplified from the Tn*5*Km, Tn*5*TcGFP and Tn*5*Tc respectively (data not shown). To narrow the focus of study, each STM mutant was also tested in a single PCR assay to confirm its absence from the *in vivo* pool; any mutant that gave very weak or barely detectable amplification product was discarded from the study. From these studies, we narrowed the screening to 214 attenuated mutants.

Analysis of the distribution of STM mutants

The miniTn*5* insertion end-points could be determined by DNA sequencing for 160 of the 214 avirulent strains. The *P. aeruginosa* strain PAO1 unique circular chromosome has been recently sequenced (Stover *et al*., 2000). As depicted in Fig.1, 148 of the 160 insertion points could be mapped to a specific PA position, and insertions were found to be evenly distributed around the chromosome. We also found contiguous insertions in at least two genes at six PA positions.

The 160 mutants represent transposition events near or into 148 distinct open reading frames (ORFs), the bioinformatics analysis of which indicated proteins from almost all functional classes. As depicted in Fig. 2, several STM insertions were found in genes

expressing hypothetical, unknown, unclassified proteins (42.6% of the total of 148 STM or 67 insertions), in proteins implicated in the transport of small molecules (9.5%, 14), (eight putative enzymes 8,2%, 12), secreted factors (4.8%, 7) and transcriptional regulators (4.8%, 7). Few insertions were found in genes coding for classes of protein involved in adaptation and protection (1.4%, 2), amino acid biosynthesis and metabolism (2.7%, 4), carbon compound catabolism (1.4%, 2), cell division (0.7%, 1), cell wall/lipopolysaccharide (LPS) biosynthesis (0.7%, 1), central intermediary metabolism (2%, 3), chaperone and heat shock (0.7, 1), etc. Curiously, no insertions were found in genes coding for antibiotic resistance, susceptibility, biosynthesis of co-factors, chemotaxis, in typical membrane proteins and in protein secretion and export apparatus. We noted that a few STM mutants contained multiple but distinct insertions at nine PA positions as shown in parenthesis in Tables 1 and 2. A close inspection of the 148 STM PA mutant strains listed in Table 1 and in Table 2 by PSORT confirmed insertions in genes encoding proteins localized to the cytoplasm (45.9%, 68), the inner membrane (31.1%, 46), the periplasm (3.4%, 5), the outer membrane (4.7%, 7) and unknown (14.9%, 22). Overall, the number of attenuated STM mutants represents approximately 2 % of the total 7968 strains that we screened.

Detailed analysis of STM mutants

The 148 mutants attenuated in the rat lung were also tested for growth in minimal media (M9 plates). Table 1 lists seven STM auxotroph mutants that could not grow on minimal media. More importantly, Table 1 lists 11 STM mutants with insertions in genes known to be important for virulence of *P. aeruginosa*. We will now refer to STM mutant strains by their PA position insertions as their STM name as shown in Tables 1-3. Bacterial cellular processes essential for *in vivo* survival and known to be crucial for *P. aeruginosa* pathogenesis are intimately involved in motility and attachment such as pili and type IV fimbriae. The three mutants STM4528, STM410, STM4554 (Table 1), which were strongly attenuated *in vivo,* had insertions in *pilD*, *pilI* and *pilY1*, respectively. *P. aeruginosa* is notorious for the copious amounts of alginate and mucus it produces in the lungs of cystic fibrosis patients. Thus, the recovery of attenuated STM762 and STM4446 strains carried mutations in *algU*, *algW* and in *mucC*, the functions of which are closely associated with

alginate biosynthesis, and its secretion and transcriptional regulation are highly relevant. Furthermore, as summarized in Table 1, we found that strains STM1248, STM3478, STM3831, STM5112 and STM5449 carry insertions in a plethora of secreted factors and toxins from *P. aeruginosa* known to be critical in virulence including an alkaline protease (STM1248), a rhamnosyl surfactant (STM3478), an amino peptidase (STM3831), an esterase (STM5112) and a glycosyl transferase (STM5449) involved in LPS biosynthesis.

The remaining 130 STM strains including the information on the gene disrupted in each and the functional classification based upon the four classes defined for *P. aeruginosa* at http://www.pseudomonas.com and updated by BLAST searches are available on our web site at http://rclevesque.rsvs.ulaval.ca/. Several genes found to be essential for lung infection have also been identified by other high-throughput screening methods such as microarrays and transciptome analysis (Whiteley *et al*., 2001; Firoved and Deretic, 2003; Schuster et *al*., 2003; Wagner *et al*., 2003; Wolfgang *et al*., 2003).

Determination of virulence defects and screening in another infection model

After confirming the attenuation of virulence with three rounds of screening in the rat lung, we evaluated whether the 148 STM strains had defects in the expression of known virulence factors, and if they could be categorized further by *in vivo* screening in the alternative model host *Drosophila melanogaster*. All 148 STM strains were tested for the production of biofilm, twitching, motility, swarming and attachment, the capacity to produce extracellular enzymes including proteases and lipase and the ability to kill flies. From the 148 strains tested, Table 2 summarizes the data for 36 that were defective in the production of known virulence factors. Of those, the majority was defective in the swimming, swarming phenotype (20 STMs), and several were found to have insertions in hypothetical or unknown proteins (14 STM strains). The eight STM mutants highly attenuated in the fly model (≤15% lethality) mostly had insertions in amino acid, nucleotide and central metabolism, except for STM410 (*pilI*) and STM5441 (hypothetical, but affecting pyoverdine overproduction).

Identification of STM151 as a probable tonB receptor required for biofilm formation

Nine STM mutants with defects in small molecule transporters were identified from our screen (see the data available at http://rclevesque.rsvs.ulaval.ca/). Of these, STM151 putatively codes for a probable outer membrane TonB-dependent receptor with 43% similarity to PupB of *Pseudomonas putida.* We used microtitre dishes made of polyvinylchloride (PVC) carefully and systematically to analyze the ability of these nine strains to form a biofilm on an abiotic surface (O'Toole and Kolter, 1998). Although the growth in minimal M9 medium was comparable to the wild-type strain PAO1, staining of the biofilm with crystal violet indicated that the STM151 mutant was clearly identified as defective in biolfim formation (Fig. 3). The PA0151 is a homologue of *P. putida* PupB, and part of a simple putative two gene operon comprising PA0150 and PA0151. As depicted in Fig. 4, it is highly unlikely that the downstream *pcaQ* and the two upstream genes separated by intergenic spacers are co-transcribed. Because STM151 has an insertion in the second gene of the two-gene operon, polar effects can be excluded. Thus, PA0151 gene is essential for *in vivo* maintenance presumably via its role in biofilm formation.

Identification of STM2895 with a defect in a hypothetical protein required for extracellular proteases

Pseudomonas aeruginosa secretes a variety of proteases into the extracellular medium including two phospholipases C, a lipase, esterases and an alkaline protease that all play complementary roles as virulence factors by hydrolysis of lung surfactant and by inducing the release of an inflammatory mediator from human platelets (Wilhelm *et al*., 1999). As shown in Fig. 5, strain STM2895 was found to have a major defect in protease production. The relative amount of elastolytic activity for STM2895 was 4.5% compared with the activity from the wild-type PAO1 strain. The gene PA2895 is under the section hypothetical protein with an unknown function and localization. Bioinformatics analysis of the genomic region encoding PA2895, as shown in Fig. 4, clearly indicated that it would be localized at the end a small operon. Therefore, insertion of a miniTn*5* into PA2895 is

unlikely to cause any polar effect, and its function can clearly be implicated in protease production.

STM4491 codes for a hypothetical protein required for swarming

The STM4491 encodes a hypothetical conserved protein putatively located in the inner membrane (a low 2.3 probability by PSORT) having 61% similarity with YfaA of *Escherichia coli*. As shown in Fig. 5, inactivation of PA4491 completely abolished the swarming and swimming capacity of STM4491. Inspection of gene organization in Fig. 5 confirmed that PA4491 is part of a small two gene operon with no predicted polarity.

Identification of STM1863 as a modA periplasmic precursor required for motility

The strain STM1863 contains an insertion in a gene coding for a periplasmic protein (PSORT of 9.4) implicated in the transport of small molecules and with 82% similarity with ModA of *Azotobacter vinelandii*. The protein can be classified as a probable multidrug efflux system component encoding a probable RND efflux transporter. As shown in Table 2 and in Fig. 5, inactivation of ModA completely eliminates the motility of strain STM1863. Examination of the genomic context in Fig. 4 confirmed that PA1863 is part of a three-gene organization including PA1861 and PA1862. We have shown previously that PA1863 can be partly complemented in *trans* (Lehoux *et al*., 2002) and the STM1863 strain contains an insertion with a polar effect.

Discussion

The vast knowledge accumulating from the completed sequences of bacterial genomes has increased the interest in essential genes and in bacterial pathogenesis as tools to develop new antibacterial treatments and vaccines. In addition, the development of new technologies for high-throughput analysis *in vivo* such as STM, IVET and differential display, *in vitro* analysis of proteome, transcriptome and chip technology for DNA and proteins should rapidly identify promising antibacterial targets for the synthesis of new generation of antibiotics and inhibitors. Recently, we have used the promising approach of genomics and essential genes coupled to the mix-and-split combinatorial chemistry approach and phage display to develop a combinatorial enzymatic assay for the screening of new bacterial cell wall inhibitors and peptides (El Zoeiby *et al*., 2003a, b).

To support this approach, there is a need of knowledge on genes and their products assumed to be essential *in vivo* during an infection, which would permit their use in antibacterial drug development and vaccines. Here, we present extensive data on a STM screen comprising 7968 STM mutants that should expand the knowledge on *P. aeruginosa* genes essential for virulence in a rat model of chronic lung infection and in the fly *Drosophila*. Interestingly, stringent criteria for the selection of STM mutants retained approximately only 3 % (when calculated using 5570 ORFs) of STM strains screened in the rat lung by PCR. It would be highly unlikely that all known virulence genes could be obtained by an STM screening using 7968 clones for a 6.3 Mb genome. Indeed, a full coverage of the 6.3 Mb for the PAO1 strain would require at least a seven- to eightfold genome equivalent for full coverage of the 5570 ORFs. This would necessitate ≈ 55 776 STM clones. Such a large number of mutants would represent a vast amount of work and a collection of 775 rats to do the actual screening.

Transposon mutagenesis has certain theoritical limitations because of unidentified hot-spots in the targeted chromosome, essential genes giving a lethal phenotype and instability of insertions. Also, there are now several *P. aeruginosa* strains available, such as PAO1, PAK and PA14, in which the degree of virulence has been studied in several hosts. The PA14 genome has been sequenced recently (S. Lory and F. Ausubel, Harvard University, personal communication), and the only noted differences from the PAO1 sequence are a

number of so-called pathogenicity islands identified. Except for these differences, both genomes are remarkably similar. Hence, there is an indication that an STM study of the PA14 strain would yield information on additional genes essential *in vivo*.

Stability, frequency and multiplicity of transposon insertions in the pyoverdin region of PAO1 have been studied previously using Tn*5* and a miniTn*10*. In PAO1, 13.1% of mutants were Pvd$^-$, and both antibiotic resistances were rapidly lost; the Pvd$^-$phenotype was stable, and the Tn*5* could not be detected by hybridization (Cornelis *et al.,* 1992). A priori, there is no indication that certain regions of the genome cannot accept miniTn*5* transposon insertions and that these insertions would be more stable in PA14 than in PAO1. Except for essential genes, one would expect an insertion in all ORFs of PAO1. Work done recently by C. Manoil and M. Jacobs (pers. comm.) at the University of Washington agrees with this point but gives no indication for the stability for the mutants obtained. The collection of 45 000 PAO1 insertion mutants as well as additional data are available at http://www.genome.washington.edu/UWGC/index.cfm.

We developed a 72-tag multiplex PCR STM, which is more stringent than the classical hybridization approach for screening, and defined strict conditions for the *in vivo* selection process. Indeed, we have coupled the multiplex PCR for initial screening to a single confirming PCR and discarded clones that gave a weak or barely detectable amplification product, a procedure that would be difficult to achieve with hybridization because of the inherent background of colony dot blots. Typical screens by STM hybridization yield from 1% to 10% attenuated strains (Lau et al., 2001; Hava and Camilli, 2002; Merrell *et al*., 2002; Kurz *et al*., 2003). It has been suggested that two critical factors contribute to the number of STM mutants obtained. The insertion of a transposon into a locus can cause polar mutations and presumably inactivate downstream genes that are co-transcribed, thus augmenting the number of attenuated strains. The second major factor depends on the animal or cell model used for *in vivo* screening. For the second criteria, a strategy needs to be defined in order to consider the power and the limitations of the animal models used and to decide the number of rounds of STM screening and the number of animals to be used.

In mammals such as the rat and mouse, several studies of bacterial pathogens have been done by STM. Merrell *et al*. (2002) have designed strategies and scenarios to evaluate the

complexity of input strains when attempting to identify novel factors of colonization and acid tolerance of *Vibrio cholerae*. Elegant studies done on the identification of large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors have also determined that colonization bottlenecks exist (Hava and Camilli, 2002). In animal infection, the population of bacteria that can initially survive and begin to multiply is restricted to those that are able to overcome certain barriers during transit to the infect site, a phenomenon referred to as the colonization bottleneck. In this study, the rationale that we used was to limit the tag complexity to 24 and use the power of bacterial genetics phenotypic selection to obtain a collection of 72 mutants per pool selected using the miniTn*5* markers Km, Tc and GFP. Also, we used a defined inoculum of *Pseudomonas* embedded in agar beads, which presumably eliminates this bottleneck. There is also a precedent for limiting the number of tags used. When *Salmonella typhimurium* was inoculated into the peritoneal cavities of mice, pools of 96 different mutants gave reproducible hybridization signals after 3 days of infection, whereas 192 did not (Unsworth and Holden, 2000). Problems with the complexity of the pool tags have also been described for *V. cholerae*, in which it was necessary to reduce the complexity of the orally inoculated pool to 48 strains to give reproducible results (Chiang and Mekalanos, 1998). Hence, mammalian models of infection using bacteria are complex and cannot necessarily reproduce faithfully all the conditions of the infection. Several parameters need to be considered: i) the number of different mutant strains to be used in a pool; ii) the route of administration, the dose for the inoculum and the incubation period; iii) the use of different animal hosts for screening the same pool of STM mutants. The inoculum size necessary to initiate an infection will determine the complexity of mutants pooled. In fact, each mutant in a defined input pool has to be in a sufficient cell number to initiate infection. The inoculum size must not be too high, resulting in the growth of mutants that would otherwise not have been detected. At higher doses, the immune system may be overwhelmed and the animals die of shock (Unsworth and Holden, 2000).

Other important parameters in STM include the route of inoculation and the time-course of a particular infection. Also, certain gene products that are directly or indirectly important for initiation or maintenance of the infection may be niche dependent or expressed specifically in certain host tissues only. For *P. aeruginosa*, the more virulent strains PA14

and PAO1 have been screened in a repertoire of hosts including plants such as *Arabidopsis* and lettuce, the amoeba *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the wax moth and the fly *Drosophila melanogaster* (Rahme *et al*., 1997; Mahajan-Miklos *et al*., 1999; Cosson *et al*., 2002; Lau *et al*., 2003). To our knowledge, the only common genes found in these systems and reported here are those listed in Table 1, such in *algU* and *pilI*, and in Table 2 for the eight *Drosophila*-specific STM. In these various models, if the duration of the infection in STM *in vivo* selection is short, genes important for establishment of the infection will be found and, if the duration is long, genes important for maintenance of infection will be identified. As STM is used and parameters are better defined in different models of infection, several routes of inoculation and different animal models can be used for the same organism studied by STM.

Bacterial communication via quorum sensing (QS) has been reported to be important in the production of virulence factors, antibiotic sensitivity, and biofilm development. Two QS systems, known as the *las* and *rhl* systems, have been identified previously in the opportunistic pathogen *P. aeruginosa*. High-density oligonucleotide microarrays for the *P. aeruginosa* PAO1 genome were used to investigate global gene expression patterns modulated by QS regulons (Wagner *et al.,* 2003). Conservatively, 616 genes showed statistically significant differential expression ($P \leq 0.05$) in response to the exogenous autoinducers and were classified as QS regulated. A total of 244 genes were identified as being QS regulated at the mid-logarithmic phase, and 450 genes were identified as being QS regulated at the early stationary phase. Most of the previously reported QS-promoted genes were confirmed, and a large number of additional QS-promoted genes were identified. Importantly, 222 genes were identified as being QS repressed. Environmental factors, such as medium composition and oxygen availability, eliminated detection of transcripts of many genes that were identified as being QS regulated. As depicted in Table 2, we have identified three essential *in vivo* genes including STM1874 (hypothetical), STM1927 (MetE, methyltransferase), and PA3876 (NarK2, nitrite extrusion protein) similar to genes whose expression was identified by microarray (Wagner *et al.,* 2003). These authors identified QS-regulated genes involved in nitrogen metabolism, including the PA1861-1863 ModABC transporters and the PA3872-PA3877 implicated in nitrate respiration that we also identified by STM as listed in Table 2. We are currently analysing

the collection of STM clones described here for the production of signal molecules such as AHLs and PQS.

Transcriptome analysis for identification, timing, and signal specificity of *P. aeruginosa* quorum-controlled genes was compared to the study done here (Schuster *et al.,* 2003). As indicated in Table 2, five STM strains were identified, including STM158 (probable RND efflux transporter), STM3734 (hypothetical), STM4172 (probable nuclease), STM3284 (hypothetical), STM4692 (conserved hypothetical). Comparison of the data from the microarray analysis of ECF sigma factor AlgU (*P. aeruginosa* sigma E)-dependent gene expression in *P. aeruginosa* with our data identified PA1592 (hypothetical, *lptA*) (Firoved and Deretic, 2003). In addition to several of the typical virulence factors listed in Table 1, a novel gene found here when screening in the rat lung was identified previously in the co-ordinate regulation of bacterial virulence genes by a novel calcium-dependent adenylate cyclase-dependent signalling pathway, STM4983, a two-component response regulator (Wolfgang *et al*., 2003). However, it was apparent that the transcriptome of cAMp- and Vfr-deficient mutants indicated that numerous host- directed virulence determinants, including motility systems, attachment organelles and the type II secretory pathway proteins, of which many are found in Table 2, are co-ordinately regulated under conditions that control expression of the type III secretion systems, which represent one of the more specific host-directed bacterial virulence determinants (Wolfgang *et al*., 2003).

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Experimental procedures

Bacterial strains, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3. Unless otherwise indicated, *E. coli* strains were grown in tryptic soy broth (TSB). *P. aeruginosa* strains (PAO1 and PAO909) were grown in brain-heart infusion (BHI, Difco). When needed, these media were supplemented with 1.5% of bacto agar, ampicillin (50 µg ml^{-1}), chloramphenicol (5 µg ml^{-1}), kanamycin (50 µg ml^{-1} for *E. coli* and 500 µg ml^{-1} for *P. aeruginosa* in media with bacto agar and 300 µg ml^{-1} in liquid media), tetracycline (5 µg ml^{-1} for *E. coli* and 15-30 µg ml^{-1} for *P. aeruginosa*).

Analysis of Virulence factors

Proteases activity. The proteolytic activity of the culture supernatants was measured as described previously by (Laux *et al*., 2000). Briefly, supernatant samples (100 µl) were added to wells made by removing agar plugs in BHI agar plates containing 1% skim milk powder (Difco) and 500 µg ml^{-1} carbenicillin (to prevent growth of residual bacteria).The plates were incubated for 24 h at 37 °C. The diameter of the zone of casein hydrolysis was measured and compared with the amount of proteolytic activity from the supernatant of the wild-type strain PAO1. Elastolytic activity from spent culture supernatant was determined essentially as described previously (Beatson *et al.,* 2002).

Biofilm formation. To assess the formation of bacterial biofilm, a 96-well plate assay was performed as described previously by (O'Toole *et al*., 2000).

Pyoverdin and siderophore production. Production of pyoverdin and iron uptake siderophores was tested visually under UV light using *Pseudomonas* Agar F and P media (Difco) respectively. Plates were streak inoculated from an overnight LB culture with a loop and incubated overnight at 37°C.

Twitching motility. Twitching motility assays were performed by stabbing a colony of bacteria into the bottom of a Petri dish containing 10 ml of 1% LB agar. After incubation at 37°C for 17 h, the volume of agar was reduced with a thick stack of circular paper towels to absorb the moisture. The zone of motility was visualized by staining the agar with Coomassie brilliant blue G250 (Suh *et al*., 1999; Beatson *et al*., 2002).

Flagellar swim plates and swarming assay. P. aeruginosa swarming ability was assessed using the previously described protocol (Köhler *et al*., 2000; Parkins *et al*., 2001). Swim plates were incubated at room temperature, and swarm plates were incubated at 37°C. All plates were grown for 72 h. Each assay was performed in duplicate, and the average results are shown.

Motility. Swimming motility was tested using Bacto Motility Medium S (Difco Laboratories). The inoculation was done by puncturing 10 ml of the semi-solid media with an overnight culture of each STM mutant (Ball and Sellers, 1966).

In vitro DNA manipulations and the design of oligonucleotides as STM DNA tags

The PCR-based STM method involves designing 24 pairs of 21-mers (Table 4) synthesized as complementary DNA strands for cloning into the pUTmini-Tn*5*Km2, miniTn*5*Tc and miniTn5TcGFP plasmid vectors, and giving a collection of 72 tags. The first twelve pairs were previously described (Lehoux *et al.*, 1999), and the other twelve pairs were designed according to the same three basic rules: (i) similar T_m of 64°C to simplify tag comparisons by using one-step PCR; (ii) invariable 5' ends with higher ΔG than at the 3' end to optimize PCR amplifications; (iii) a variable 3' end for an optimized yield of specific amplification product from each tag. Tags were synthesized by MGW Biotech. Annealing reactions contained 50 pmol of both complementary oligonucleotides in 100 µl of medium salt buffer (Sambrook and Russel, 2001).

Cloning of tags into miniTn5s

Three mini Tn*5*-based transposons, pUTmini-Tn5Km2, pUTmini-Tn*5*Tet and pUTmini-Tn*5*TetGFP, were used for mutagenesis (Fellay *et al.,* 1987; De Lorenzo *et al*., 1990; Herrero *et al.,* 1990; Matthyse *et al*., 1996). The transposons are located on a R6K-based suicide delivery plasmid pUT in which the Pi protein is furnished by the donor cell; the pUT plasmid provides the IS50R transposase *tnp* gene in *cis* but external to the mobile element, conjugal transfer of which to recipients is mediated by RP4 mobilization functions in the donor (Simon *et al*., 1983). Plasmid DNA (0.04 pmol) was ligated with 1 pmol of double-stranded DNA tags in a final volume of 10 µl of T_4 DNA ligase 1X buffer containing 400 units of T_4 DNA ligase in 24 separate reactions (Sambrook and Russel, 2001). The pUTmini-Tn*5* Km2 was digested with *Kpn*I (New England Biolabs), and recombinant molecules were constructed *in vitro* by blunt-end fill-in with T4 DNA polymerase (Gibco BRL Products). The pUTminiTn5Tc and Gfp were digested with *Not*I (New England Biolabs), and recombinant molecules were constructed *in vitro* by blunt-end fill-in with Klenow (New England Biolabs). Ligated products were purified using microcon PCR (Millipore) and resuspended in 5 µl of H_2O. All the 5 µl-containing ligated products were transformed into *E. coli* S17-λ*pir* by electroporation. Transformed bacteria containing tagged plasmids were selected on TSA supplemented with 50 µg ml^{-1} ampicillin and 50 µg ml^{-1} kanamycin. Single colonies were selected, purified and screened by colony using 10 pmol of one of the oligonucleotide tags (Lehoux and Levesque, 2001) used to construct the DNA tags as a 5' primer and 10 pmol of the pUTKana2, 5'-GCGGCCTCGAGCAAGACGTTT-3'; pUTgfpR2, 5'-ATCCATGCCATGTGTAATCCC-3'; tetR1, 5'-CCATACCCACGCCGAAACAAG-3' as the 3'- primers in the transposon resistance gene. Thermal cycling conditions were for touchdown PCR using a hot-start for 7 min at 95°C, two cycles at 95°C for 1 min, temperature ramping range of 70°C to 60°C for 1 min and at 72°C for 1 min, followed by 10 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min in a DNA thermal cycler (Icycler, Bio-rad). Amplified products (10 µl) were analyzed by electrophoresis in a 1% agarose gel, 1X Tris-borate EDTA buffer and stained for 10 min in a 0.5-µg ml^{-1} ethidium bromide solution (Sambrook and Russel, 2001).

Conjugation and construction of 72 miniTn5 P. aeruginosa mutant libraries

Escherichia coli S17-λ*pir* containing the pUTminiTn*5* plasmids with tags was use as a donor for conjugal transfer into the recipient *P. aeruginosa* strain PAO1 using a ratio of one donor cell of *E. coli* with 10 recipient cells of *P. aeruginosa*. Bacterial cells were mixed and spotted as a 50 µl drop on a sterile nylon membrane placed on a non-selective BHI agar plate. Plates were incubated at 30°C for 18h. Filters were washed with 1 ml of sterile phosphate-buffered saline (PBS) to recover bacteria. Five 100 µl aliquots of the PBS solution containing transconjugants were plated on five BHI agar plates supplemented with the appropriate antibiotics. Kanamycin was used to select transconjugants with the miniTn*5*Km2 and Tc with the miniTn5Tc and miniTn*5*TcGFP. Plates were incubated overnight at 37°C. Exconjugants were selected on BHIA supplemented with Cm (5µg ml^{-1}) (Sigma Chemical) and Km (500 µg ml^{-1}) for the mini-Tn*5*Km2 or Tc (15 ml^{-1}) for the miniTn*5*Tc and miniTn5GFP. Kanamycin-resistant and ampicillin-sensitive exconjugants were arrayed as libraries of 96 clones in 96-well microtitre plates using 1.5 ml of BHI supplemented with kanamycin and appropriate antibiotics. To assemble the mutant library, one mutant from each library is picked to form 96 pools of 72 unique tagged mutants in each 2 ml well, labeled and arrayed. In a defined library, each mutant had the same tag but theoretically inserted at a different location in the bacterial chromosome.

Screening of STM mutants in the rat lung model of chronic infection

We have used the rat chronic lung infection model to screen our STM pools (Cash *et al*.1979). Female Sprague-Dawley rats of 140 to 160 g in weight were used. The animals were anesthetized using Isofluorane and inoculated into the left lobe of lungs with 100 µl of a suspension of agar beads containing 10^4, 10^5 or10^6 bacterial cells (the *in vitro* pool). After 7 days, lungs were removed from sacrificed rats and homogenized tissues were plated on BHIA supplemented with chloramphenicol. Colonies recovered after *in vivo* selection were used for multiplex PCR (the *in vivo* pool) as described previously (Lehoux and Levesque, 2000). Ninety-six pools of 72 mutants forming a collection of 6912 mutants and 96 pools of 11 mutants were screened. We used an infecting dose of 10^6 bacteria per animal. After 7

days of infection, an average of 10^6 bacteria was recovered from the lung of each animal. To identify mutants not recovered after the *in vivo* passage, screening was done by PCR using bacterial colonies. Mutants which gave no amplification products by multiplex PCR after the *in vivo* selection were re-tested by single PCR. Colony PCR amplification products obtained from the *in vitro* pool was compared to the *in vivo* pool. Mutants which gave positive results from the *in vitro* pool and absent from the *in vivo* pool were kept for further analysis.

Screening of tagged mutants by multiplex PCR

Detection of mutants was done by performing multiplex colony PCR in 50µl reaction volumes containing10 µl of boiled bacterial colony in 100 µl of TE (10 mM Tris pH 7,4; 0,1 mM EDTA); 5 µl of 10X HotStart *Taq* polymerase (Qiagen) reaction buffer (15 mM $MgCl_2$; 200 µM of each dNTPs; 10 pmoles of one of the 21-mers). The 21-mers numbered 1 to 24 in Table 3 were used as a first primer in combination with 10 pmoles of each pUTKana2, tetR1 and pUTgfpR2 primers for selective amplification. HotStart *Taq* polymerase 2.5 U (Qiagen, Mississauga, Ont, Canada) was used in each PCR. Amplification conditions were: hot start 15 min. at 95°C, 2 cycles at 95°C for 1 min., 65 to 55°C for 1 min., and at 72°C for 1 min. followed by 10 cycles at 95°C for 1 min., 55°C for 1 min., 72°C for 1 min. (Touchdown PCR) in a ICycler (BioRad). Amplified tags from *in vivo* pool were compared with amplified tags from *in vitro* pool. Mutants that gave PCR amplification products from *in vitro* pool and not from *in vivo* pool were purified and kept for further analysis. For confirmation, the PCR reaction was repeated for each putative STM mutant. These STM mutants were pooled, and used for a second round of *in vivo* screening.

Cloning and analysis of disrupted STM gene mutants selected.

Chromosomal DNA from the STM mutants was prepared using the QIAGEN genomic DNA extraction kit as described by the manufacturer. *C*hromosomal DNA (1µg) was digested with *Pst*I giving DNA fragments ranging in size from 1 Kb to 6 Kb. Digested

chromosomal DNA was cloned into pTZ18R (Amersham Pharmacia Biotech). Ligations were performed using 1 μg of digested chromosomal DNA mixed with 50 ng of digested pTZ18R in 20 μl of 1X T_4 DNA ligase buffer with 40 units of T_4 DNA ligase. Ligated products were incubated overnight at 16°C, and purified using the microcon PCR (Millipore) as described by the manufacturer. The recombinant plasmid was resuspended in 5 μl of H_2O used for electroporation into *E. coli* DH5α. Bacterial clones were purified and analyzed for plasmid content with the QIAGEN midi preparation kit as described by the manufacturer.

Maintenance of Fly stocks

D. melanogaster Oregon-R stocks were maintained on standard yeast extract-agar-sucrose-cornmeal medium at 24°C.

Fly infection assays

Drosophila infections were performed as previously described (Lau *et al.,* 2003). All experiments used healthy, 4- to 7-day-old adult male *D. melanogaster* Oregon-R flies. As controls, we found that aseptic injuries by pricking flies on the dorsal thorax with a 10-μm-diameter needle (Ernest F. Fullam, Inc., Latham, New York) which had previously been dipped into 10 mM $MgSO_4$ did not cuase death. After being dipped into a bacterial suspension containing 5×10^7 CFU/ml from an early stationary phase (optical density at 600 nm, 3.0) bacterial culture, the needle was used to infect flies with 10 to 100 *P. aeruginosa* cells/fly. Fly lethality was determined for groups of ≥50 infected flies for up to 72 h at 24°C. Flies that died within 12 h after infection were excluded. Lethality studies were repeated at least three times. (For bacterial growth studies, infected flies were collected at indicated time intervals, ground in 10 mM $MgSO_4$, and plated onto Luria-Bertani agar plates supplemented with appropriate antibiotics, with colony counts scored after 14 to 16 h at 37°C. Statistical analysis of fly survival was performed using Student's t-test to compare the difference between two groups and one-way analyses of variance (ANOVA). A significant difference will be considered for $P<0.05$.

Bioinformatics analysis

The DNA sequences were analyzed with the University of Wisconsin Genetics Computer Group software version 11 available through the RSVS Biocomputing facilities, Université Laval. The information obtained from the sequencing data by DNA and protein BLAST and NCBI searches was compared with the database available for *P. aeruginosa* at http://www.pseudomonas.com.

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Figure Legends

Fig.1. Schematic depiction of the chromosomal locations of all 148 open reading frames isolated with transposon insertions that resulted in reduced infection when using as using as animal host a rat model of chronic respiratory infection. The gene designation and predicted initiation start sites are given according to the annotation of the complete 6.3 Mb *P. aeruginosa* strain genome available at http://pseudomonas.com.

Fig. 2. The distribution of 148 STM genes inactivated after *in vivo* passage in the lung for 7 days. The 26 classes of genes used are those from various bacterial genome sequencing projects available at http://www.tigr.com and at http://pseudomonas.com.

Fig.3. Analysis of biofilm formation using a collection of STM strains essential for lung infection. The biofilm formation of the wild-type PAO1 and the STM151 defective mutant represented by open bars was quantitated over 24 h by using microtiter dish assay, as reported elsewhere (O'Toole and Kolter, 1998). The A_{600} value represents crystal-violet stained bacteria attached to the walls of the microtiter dish and is an indirect measure of the biofilm formed. The data represents four experiments, each performed in triplicate.

Fig.4. Genomic organization of the four STM151 (Probable TonB receptor), STM2895 (Hypothetical), STM4491 (Conserved, hypothetical) and STM1863 (Molybdate binding precursor) mutants via miniTn*5* insertions in the chromosome of *P. aeruginosa* strain PAO1. The gray boxes inside each predicted labeled ORF indicate the insertion and orientation of the minitransposon. The name of each inactivated ORF, PA0151, PA2895, PA4491 and PA1863 is shown below each locus. Arrows and direction of transcription

represent ORFs as they appear in the annotation of the *P. aeruginosa* genome database at http://www.pseudomonas.com.

Fig.5. Phenotypic analysis of *P. aeruginosa* strains STM2895 (Hypothetical), STM4491 (Conserved, hypothetical) and STM1863 (Molybdate binding precursor) for the expression of known virulence factors. A, Evaluation of extracellular protease production from the wild-type strain PAO1. Serial fold dilutions of the supernatant are presented (*see experimental procedures for details*) and B, starting at 8 o'clock counter clock wise for strain STM410, a PilD defective mutant (Number 2 in Table 1, under the group of known virulence factors); strain STM0073, a probable ATP-binding ABC transporter 59% homologous to YbjZ of *E. coli* (Number 130 in Table 2 under the class of transport of small molecules; STM4488, a conserved hypothetical defective in swarming (Number 49 in Table 2 and in Table 3 and STM2895, an hypothetical unclassified also defective in protease production as shown here (Number 97 in Table 2 and in Table 3); C, Analysis of swarming for the *P. aeruginosa* wild-type and D, STM4491 strains. STM4491 has an insertion in PA4491 as depicted in Fig. 4 and shows no swarming capacity. Colonies of the wild-type and mutant STM4491 from a fresh agar plate were inoculated by toothpick into the middle of the swarm plates and incubated for 24 h (Kohler *et al*., 2000; Parkins *et al.,* 2001); E, Evaluation of the twitching motility for STM1863 (left) compared to the wild-type PAO1 strain (right). STM1863 codes for ModA (Number 133 in table 2 and in Table 3). Twitching motility was evaluated as described in Experimenatl procedures (Beatson *et al.,* 2002).

Table 1. *P. aeruginosa* genes essential for lung infection known previously as virulence factors and auxotrophs

	Strain name	STM Name PA Position	Gene Name[a]	Description	Homologue[b]	Localization[c]	Reference[d]
	Known virulence factors						
1	G76T9	PA4528	*pilD*	Motility & attachment	100% PilD *P. aeruginosa*	IM (10)	Beatson *et al*., 2002
2	33T24K	PA0410	*pilI*	Motility & attachment	100% PilI *P. aeruginosa*	C (7.3)	Kohler *et al*, 2000
3	6T1K	PA4554	*pilY1*	Motility & attachment	100% PilY1 *P. aeruginosa*	IM (2.3)	Choi *et al.,* 2002
4	84T6K	PA0762	*algU*	Sigma factor	100% AlgU *P. aeruginosa*	C (7.3)	Wagner *et al*., 2003
5	33T1K	$PA4446_{(2)}$	*algW*	Secretion of alginate	100% AlgW *P. aeruginosa*	P (4.6)	Firoved and Deretic, 2003
6	G17T12	PA0765	*mucC*	Transcription regulator	100% MucC *P. aeruginosa*	U (2)	Govan and Deretic, 1996
7	49T23T	PA1248	*aprf*	Alkaline protease	100% AprF *P. aeruginosa*	OM (10)	Wilhelm *et al*., 1999
8	11T20T	$PA3478_{(2)}$	*rhlB*	Rhamnosyl transferase	100% RhlB *P. aeruginosa*	C (7.3)	Davey *et al.,* 2003
9	14T19K	PA3831	*pepA*	Leucine amino peptidase	100% PhpA *P. aeruginosa*	C (7.3)	Govan and Deretic, 1996
10	35T1G	PA5112	*estA*	Esterase	69% YtrP *P. putida*	OM (10)	Govan and Deretic, 1996
11	15T21T	PA5449	*wbpX*	Glycosyl transferase	100% WpbX *P. aeruginosa*	C (7.3)	Daniels *et al*., 2002
	Auxotrophs						
12	14T11K	PA2876	*pyrF*	Orotidine decarboxylase	100% PyrF *P. aeruginosa*	C (7.3)	
13	G49T2	PA3735	*thrC*	Threonine synthase	100% ThrC *P. aeruginosa*	C (7.3)	

14	G79T4	PA3763$_{(2)}$	*purL*	Phosphoribosyl synthase	78% PurL *E. coli*	C (7.3)
15	G61T10	PA4854	*purH*	Phosphoribosyl transferase	78% PurH *E. coli*	C (7.3)
16	G75T1	PA4855$_{(2)}$	*purD*	Phosphoribosyl ligase	81% PurD *E.coli*	C (7.3)
17	40T15K	PA5131	*pgm*	Phosphoglycerate mutase	86% Pgm *P. syringae*	C (9.8)
18	33T 8K	PA5437		Transcription regulator	62% CbbRI *R. capsulatus*	C (9.8)

a, Indicates the gene name assigned by the annotation and sequencing group at the http://www.pseudomonas.com internet site.

b, Indicates the name of the protein homologue, when available with recent BLAST analysis and updating data from the http://www.pseudomonas.com site. There are 4 classes; 1, Function experimentally demonstrated in *P. aeruginsa*; 2, Function of highly similar gene experimentally demonstrated in another organism (and gene context consistent of pathways its involved in, if known; 3, Function proposed based on presence of conserved amino acid motif, structural feature or limited sequence similarity to an experimentally studied gene; 4, Homologs of previously reported genes of unknown function, or no similarity to any previously reported sequences.

c, Protein localization was determined using PSORT. Abbreviations: C, cytoplasmic; IM, inner membrane; P, periplasm; OM, outer membrane; U, Unknown (Gardy *et al*., 2003).

d, References are given for genes which have been previously assigned a role in virulence.

Table 2. *Pseudomonas aeruginosa* genes essential for lung infection

	Strain	STM Name PA Position	Gene name[a]	Description	Homologue[b]	Localisation	References[a]
	Adaptation, protection						
19	71T1G	PA4427	*sspB*	Stringent starvation B	59% SspB *E. coli*	C (7.3)	
20	82T10G	PA4915		Probable chemotaxis transducer	56% PctB *P. aeruginosa*	IM (9)	
	Amino acid biosynthesis and Methionine metabolism						
21	35T6T	PA1927	*metE*	Homocysteine S methyltransferase	74% MetE *E. coli*	C (7.3)	Wagner *et al.,* 2003
22	15T17K	PA0895	*aruC*	Succinyl glutamate dehydrogenase	100% AruC *P. aeruginosa*	C (7.3)	
23	G15T6	PA0447	*gcdH*	Glutaryl-CoA dehydrogenase	78% GcdH *H. sapiens*	C (7.3)	
	Carbon compound catabolism						
24	5T15T	PA0552	*pgk*	Phosphoglycerate kinase	82% Pgk *E. coli*	C (9.8)	
	Cell division						
25	35T22G	PA0375	*ftsX*	Cell division	53% FtsX *E. coli*	IM (9)	
	Central intermediary metabolism						
26	12T14T	PA3524	*gloA1*	Lactoyl glutathione lyase	85% GloA1 *N. meningitidis*	C (7.3)	

27	33T19T	PA4626	*hprA*	Glycerate dehydrogenase	61% HprA *M. extorquens*	C (9.8)	
28	33T14G	PA4024	*eutB*	Ethanolamine ammonia-lyase	80% EutB *R. erythropolis*	C (7.3)	
	Chaperones & heat shock						
29	G38T4	PA1596	*htpG*	Chaperones & heat shock	78% HtpG *E. coli*	C (9.8)	
	DNA replication, recombination, modification and repair						
30	62T7G	PA3620	*mutS*	DNA mismatch repair	93% MutS *A. vinelandii*	C (7.3)	
31	12T18K	PA3642	*rnhB*	Ribonuclease HII	79% RnhB *E. coli*	C (7.3)	
32	G25T4	PA4172		Probable nuclease	52% exonuclease III *E. coli*	C (7.3)	Schuster *et al.,* 2003
	Energy metabolism						
33	12T11T	PA1174	*napA*	Nitrate reductase	84% NapA *E. coli*	P (9.21)	
34	11T21K	PA2639	*nuoD*	NADH dehydrogenase I	89% NADH dehydrogenase I chain C, D *E. coli*	C (7.3)	
35	15T19G	PA2642	*nuoG*	NADH dehydrogenase I chain G	78% NADH dehydrogenase I chain G *E. coli*	C (7.3)	

36	12T16K	PA2998	*nrqB*	Na+-translocating NADH:ubiquinone oxidoreductase	76% NqrB *V. alginolyticus*	IM (9)	
	Fatty acid and phospholipid metabolism						
37	15T15K	PA3299	*fadD1*	Long-chain-fatty-acid ligase	72% FadD *E. coli*	C (9.8)	
	Hypothetical, Unclassified, Unknown						
38	36T13G	PA0077$_{(3)}$	Conserved (C) hypothetical	Hypothetical, unclassified, unknown	40% IcmF *L. pneumophila*	U (2)	
39	11T11G	PA0454	C, hypothetical	Hypothetical, unclassified, unknown	58% YccS *E. coli*	IM (9)	
40	71T10G	PA1451	C, hypothetical	Hypothetical, unclassified, unknown	67% YgcC *E. coli*	IM (9)	
41	74T15 G	PA1874	C, hypothetical	Hypothetical, unclassified, unknown	44% hemagglutinin *R. solanacearum*	OM (7.4)	Wagner *et al.,* 2003
42	42T23G	PA2462	C, hypothetical	Hypothetical, unclassified, unknown	38% hemagglutinin *R. solanacearum*	OM (10)	
43	27T23K	PA3286	C, hypothetical	Hypothetical, unclassified, unknown	69% 3-oxoacyl synthase*C. crescentus*	C (7.3)	
44	78T13G	PA3291	C, hypothetical	Hypothetical, unclassified, unknown	82% PA3292 *P. aeruginosa*	U (2)	
45	G34T11	PA3683	C, hypothetical	Hypothetical, Unclassified, unknown	57% vc1165 *V. cholerae*	C (7.3)	

46	82T15T	PA3922	C, hypothetical	Hypothetical, unclassified, unknown	84% PA3421 *P. aeruginosa*	IM (2.3)
47	G56T2	PA4115	C, hypothetical	Hypothetical, Unclassified, unknown	71% vc0899 *V. cholerae*	C (7.3)
48	21T4T	PA4473	C, hypothetical	Hypothetical, unclassified, unknown	59% YjgA *E. coli*	C (7.3)
49	G78T12	PA4488	C, hypothetical	Hypothetical, Unclassified, unknown	64% YfaQ *E. coli*	IM (2.3)
50	G36T11	PA4489	C, hypothetical	Hypothetical, unclassified, unknown	68% YfaS *E. coli*	IM (2.3)
51	G30T12	PA4491	C, hypothetical	Hypothetical, Unclassified, unknown	61% YfaA *E. coli*	IM (2.3)
52	G31T1	PA4543	C, hypothetical	Hypothetical, Unclassified, unknown	68% YfiH *E. coli*	U (2)
53	19T17G	PA4634	C, hypothetical	Hypothetical, unclassified, unknown	48% Rv3226c *M. tuberculosis*	U (2)
54	G63T12	PA5201	C, hypothetical	Hypothetical, unclassified, unknown	77% YhgF *E. coli*	C (7.3)
55	11T20G	PA5295	C, hypothetical	Hypothetical, unclassified, unknown	54% Y4LL *Rhizobium sp.*	U (2)
56	G19T12	PA5441	C, hypothetical	Hypothetical, unclassified, unknown	44% ypo0870 *Y. pestis*	IM (2.3)
57	35T5G	PA5485	C, hypothetical	Hypothetical, unclassified, unknown	61% Alanine amidase *Y. pestis*	C (9.8)
58	71T16T	PA3598	C, hypothetical	Hypothetical, unclassified, unknown	52% Q9xa70 *S. coelicolor*	C (7.3)
59	35T5K	PA4564	C, hypothetical	Hypothetical, unclassified, unknown	85% CreA *E. coli*	IM (2.3)

60	35T12G	PA1981	C, hypothetical	Hypothetical, unclassified, unknown	53% Q52118 *E. stewartii*	IM (2.3)	
61	49T13G	PA2457	C, hypothetical	Hypothetical, unclassified, unknown	50% Q8xpq3 *R. solanacearum*	U (2)	
62	82T10T	PA2684	C, hypothetical	Hypothetical, unclassified, unknown	42% RhsC *E. coli* O157:h7	C (7.3)	
63	35T18G	PA2972	C, hypothetical	Hypothetical, unclassified, unknown	71% YceF *S. typhimurium*	C (7.3)	
64	12T11K	PA3611	C, hypothetical	Hypothetical, unclassified, unknown	78% Q9kht5 *P. putida*	IM (2.3)	
65	35T9G	PA3756	C, hypothetical	Hypothetical, unclassified, unknown	67% mlr7497 *R. loti*	IM (2.3)	
66	43T13G	PA4308	C, hypothetical	Hypothetical, unclassified, unknown	67% YjgR *E. coli*	C (7.3)	
67	48T9G	PA4620	C, hypothetical	Hypothetical, unclassified, unknown	52% Hydroxy benzoyl-CoA reductase *T. aromatica*	C (7.3)	
68	53T4T	PA4692	C, hypothetical	Hypothetical, unclassified, unknown	75% r01383 *R. meliloti*	U (2)	Schuster *et al*., 2003
69	12T17K	PA4852	C, hypothetical	Hypothetical, unclassified, unknown	72% YhdG *E. coli*	C (7.3)	
70	35T4K	$PA5078_{(2)}$	C, hypothetical	Hypothetical, unclassified, unknown	82% mdoG gene *E. coli*	P (9.4)	
71	50T13G	PA5083	C, hypothetical	Hypothetical, unclassified, unknown	68% r00736 *R. meliloti*	C (7.3)	
72	12T13T	PA3893	C, hypothetical	Hypothetical, unclassified, unknown	71% PA0875 *P. aeruginosa*	IM (9)	

73	62T20G	PA4929	C, hypothetical	Hypothetical, unclassified, unknown	56% YhcK *B. subtilis*	IM (9)
74	15T5K	PA4011	C, hypothetical	Hypothetical, unclassified, unknown	61% DedA *S. typhi*	IM (9)
75	35T19K	PA4317$_{(2)}$	C, hypothetical	Hypothetical, unclassified, unknown	35% vc1429 *V. colerae*	IM (9)
76	41T10G	PA4338	C, hypothetical	Hypothetical, unclassified, unknown	42% acyltransferase *S. thermotolerans*	IM (9)
77	85T8K	PA5114	C, hypothetical	Hypothetical, unclassified, unknown	63% rsc0786 *R. solanacearum*	IM (9)
78	2T12K	PA3826	C, hypothetical	Hypothetical, unclassified, unknown	49% vc2498 *V. cholerae*	IM (9)
79	27T23T	PA4834	C, hypothetical	Hypothetical, unclassified, unknown	74% y2834 *Y. pestis*	IM (9)
80	15T22G	PA0141	C, hypothetical	Hypothetical, Unclassified, Unknown	74% PvdS *M. tuberculosis*	C (7.3)
81	G10T7	PA0082	Hypothetical	Hypothetical, Unclassified, Unknown		U (2)
82	G21T11	PA0088	Hypothetical	Hypothetical, Unclassified, Unknown		C (7.3)
83	G23T2	PA0098	Hypothetical	Hypothetical, Unclassified, Unknown		C (7.3)
84	27T4K	PA0429	Hypothetical	Hypothetical, unclassified, unknown		C (7.3)
85	30T7T	PA0561	Hypothetical	Hypothetical, unclassified, unknown		IM (9)
86	42T3G	PA0977	Hypothetical	Hypothetical, unclassified, unknown		U (2)
87	82T13G	PA2439	Hypothetical	Hypothetical, unclassified, unknown		IM (9)
88	27T4G	PA3298	Hypothetical	Hypothetical, unclassified, unknown		U (2)

89	95T24G	PA3110	Hypothetical	Hypothetical, unclassified, unknown		U (2)	
90	G73T12	PA4842	Hypothetical	Hypothetical, Unclassified, Unknown		U (2)	
91	15T6K	PA1592	Hypothetical	Hypothetical, unclassified, unknown		IM (2.3)	Firoved and Deretic, 2003
92	83T1K	PA0753	Hypothetical	Hypothetical, unclassified, unknown		IM (9)	
93	30T24G	PA0260	Hypothetical	Hypothetical, unclassified, unknown		U (2)	
94	89T16G	PA1009	Hypothetical	Hypothetical, unclassified, unknown		C (8.4)	
95	14T24T	PA1401	Hypothetical	Hypothetical, unclassified, unknown		C (7.3)	
96	11T21T	PA1471	Hypothetical	Hypothetical, unclassified, unknown		IM (2.3)	
97	1T24G	PA2895	Hypothetical	Hypothetical, unclassified, unknown		U (2)	
98	74T19T	PA4625	Hypothetical	Hypothetical, unclassified, unknown		OM (7.4)	
	Nucleotide biosynthesis and metabolism						
99	35T11G	PA0441	*dht*	Dihydropyrimidinase	95% D-hydantoinase *P. putida*	C (7.3)	
	Putative enzymes						
100	11T23K	PA0779		Probable ATP-dependent protease	61% mitochondrial *H. sapiens*	C (9.8)	
101	G91T8	PA1054		Probable NADH dehydrogenase	69% phaA *S. meliloti*	IM (10)	

102	43T7K	PA3173	Probable short-chain dehydrogenase	62% oxido reductase *V. cholerae*	U (2)
103	15T16G	PA3659	Probable aminotransferase	69% amino transferase *R. solanacearum*	C (7.3)
104	71T15G	PA3972	Probable acyl-CoA dehydrogenase	65% FadE8 *M. tuberculosis*	C (7.3)
105	82T22T	PA4434	Probable oxidoreductase	71% Tas *E. coli*	U (2)
106	82T21T	PA0298	Probable glutamine synthetase	50% YcjK *E. coli*	C (9.8)
107	G24T4	PA3001	Probable glyceraldehyde-3-phosphate dehydrogenase	76% dehydrogenase *S. roseofulvus*	C (9.8)
108	36T24G	PA3498	Probable oxidoreductase	64% benzoate dioxygenase *P. pseudoalcaligenes*	C (7.3)
109	35T20G	PA5084	D-amino acid dehydrogenase (ec 1.4.99)	55% DadA E. coli	C (7.3)
110	12T3K	PA5312	Probable aldehyde dehydrogenase	71% AldH *E. coli*	C (9.8)
111	42T19T	PA5327	Probable oxidoreductase	58% oxidoreductase *C. crescentus*	C (7.3)

Related to phage, transposon, or plasmid

112	49T24G	PA0620		Probable bacteriophage protein	80% orf20 phage phi CTX	IM (2.3)
	Secreted Factors (toxins, enzymes, alginate)					
113	15T23G	PA0041		Probable haemagglutinine	43% hemagglutinin *B. pertussis*	OM (9.7)
	Transcription, RNA processing, degradation					
114	28T21G	PA0584	*cca*	tRNA nucleotidyl transferase	71% tRNA nucleotidyl transferase *E. coli*	C (7.3)
115	12T15K	PA3308	*hepA*	RNA helicase	67% HepA *E. coli*	C (7.3)
116	G62T12	PA0455	*dbpA*	RNA helicase	69% DbpA *E. coli*	C (7.3)
	Transcriptional regulators					
117	27T9T	PA2704		Probable transcriptional regulator	54% OruR *P. aeruginosa*	U (2)
118	48T5G	PA1399		Probable transcriptional regulator	58% PA2551 *P. aeruginosa*	C (8.4)
119	12T19T	PA4659		Probable transcriptional regulator	47% YcgE *E. coli*	U (2)
	Translation, post-translational modification, degradation					
120	G20T2	PA0090		Probable ClpA/B-type chaperone	56% ClpB *Synechococcus sp*	C (7.3)
121	12T17T	PA0459		Probable ClpA/B ATP subunit	70% ClpC *B. subtilis*	C (7.3)

	Transport of small molecules						
122	35T7G	PA0605		Probable permease ABC transporter	62% MotC *A. tumefaciens*	IM (10)	
123	67T24G	PA0137		Probable permease of ABC transporter	59% ribose transporter *S. fulgidus*	IM (9)	
124	76T13G	PA1436		Probable RND efflux transporter	67% YegN *E. coli*	IM (10)	
125	35T16G	PA5167		Probable c4-dicarboxylate	90% dicarboxylate *P. stutzeri*	P (9.4)	
126	G18T2	PA0158		Probable RND efflux transporter	45% cation efflux *H. pylori*	IM (10)	Wagner *et al.*, 2003
127	96T13G	PA3876	*narK2*	Nitrite extrusion protein 2	74% NarK *E. coli*	IM (9)	Wagner *et al.*, 2003
128	11T16T	PA4887		Probable MFS transporter	47% YcaD *E. coli*	IM (9)	
129	11T4T	PA5075		Probable permease ABC transporter	72% GlnP *A. fulgidus*	IM (10)	
130	G94T2	PA0073		Probable ATP-binding ABC transporter	59% YbjZ *E. coli*	IM (9.6)	
131	76T14G	PA0151		Probable TonB-dependent receptor	43% PupB *P. putida*	OM (9.7)	
132	11T8T	PA0287		Probable sodium: solute symporter	46% PanF *E. coli*	IM (10)	
133	G13T12	PA1863	*modA*	Molybdate-binding precursor	82% ModA *A. vinelandii*	P (9.4)	Wagner *et al.*, 2003
134	40T12K	PA2408		Probable ATP-binding ABC transporter	61% MntA *F. nucleatum*	IM (8.7)	
135	23T2K	PA3607	*potA*	Polyamine transport protein PotA	64% PotA *E. coli*	C (8.9)	

	Two component regulatory systems						
136	12T8G	$PA3191_{(2)}$		Probable two component sensor	55% his kinase C. crescentus	IM (9.6)	
137	35T12K	PA1157		Probable two-component regulator	64% OmpR *E. coli*	C (9.8)	
138	48T3G	PA4983		Probable two-component regulator	63% DmsR *R. sphaeroides*	C (9.8)	Wolfgang *et al.,* 2003
139	47T21G	PA0930		Two-component sensor	68% PfeS *P. aeruginosa*	IM (9.6)	
140	15T4T	PA4886		Probable two-component sensor	56% CzcS *R. eutropha*	IM (9.7)	
	Intergenic region						
141	11T19K	45 nt PA0428	*rhlE*	Probable ATP-dependent RNA helicase	68% RhlE *E. coli*	U (2)	
142	46T13G	PA0695-0696	C, hypothetical	Hypothetical, unclassified, unknown	39% TonB *P. putida*	U (2)	
143	56T24K	83 nt PA5347	Hypothetical	Hypothetical, unclassified, unknown		U (2)	
144	48T2G	304 nt PA3284	C, hypothetical	Hypothetical, unclassified, unknown	58% Q8efg6 *S. oneidensis*	IM (2.3)	Schuster *et al.,* 2003
145	15T13G	329 nt PA0807	C, hypothetical	Hypothetical, unclassified, unknown	72% AmpD *Y. pestis*	IM (9)	
146	45T12K	37 nt PA3664	C, hypothetical	Hypothetical, unclassified, unknown	77% r00306 *R. meliloti*	U (2)	
147	12T19K	PA4763-4764		Intergenic region			
148	50T15T	510 nt PA3734	Hypothetical	Hypothetical, unclassified, unknown		C (9.8)	Schuster *et al.*, 2003

a, Indicates the gene name assigned by the annotation and sequencing group at the http://www.pseudomonas.com internet site..

b, Indicates the name of the protein homologue, when available with recent BLAST analysis and updating data from the http://www.pseudomonas.com site. There are 4 classes; 1, Function experimentally demonstrated in *P. aeruginsa*; 2, Function of highly similar gene experimentally demonstrated in another organism (and gene context consistent of pathways its involved in, if known; 3, Function proposed based on presence of conserved amino acid motif, structural feature or limited sequence similarity to an experimentally studied gene; 4, Homologs of previously reported genes of unknown function, or no similarity to any previously reported sequences.

C, References are given for genes which have been assigned a role in virulence.

Table 3. *P. aeruginosa* genes essential for lung infection defective in virulence factors and screening in *Drosophila*

	Strain name	STM Name PA Position	Gene Name	Description	Relevant characteristics
1	33T24K	PA0410	*pilI*	Motility & Attachment	Twitching, swarming, *Drosophila*
9	14T19K	PA3831	*pepA*	Leucine amino peptidase	Swarming
12	14T11K	PA2876	*pyrF*	Orotidine decarboxylase	*Drosophila*
17	40T15K	PA5131	*pgm*	Phosphoglycerate mutase	*Drosophila*
21	35T6T	PA1927	*metE*	Homocysteine methyl transferase	Swarming
24	5T15T	PA0552	*pgk*	Phosphoglycerate kinase	Lipase, pyocyanine, pyoverdine, *Drosophila*
34	11T21K	PA2639	*nuoD*	NADH dehydrogenase	Pyocyanine, motility
36	12T16K	PA2998	*nrqB*	Translocating oxido reductase	Swarming
43	27T23K	PA3286	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
47	G56T2	PA4115	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
49	G78T12	PA4488	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
50	G36T11	PA4489	C, hypothetical	Hypothetical, unclassified, unknown	*Drosophila*
51	G30T12	PA4491	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
56	G19T12	PA5441	C, hypothetical	Hypothetical, unclassified, unknown	Pyoverdine (++), *Drosophila*
59	35T5K	PA4564	C, hypothetical	Hypothetical, unclassified, unknown	Swarming

63	35T18G	PA2972	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
65	35T9G	PA3756	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
68	53T4T	PA4692	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
70	35T4K	$PA5078_{(2)}$	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
78	2T12K	PA3826	C, hypothetical	Hypothetical, unclassified, unknown	Swarming
94	89T16G	PA1009	Hypothetical	Hypothetical, unclassified, unknown	Swarming
97	1T24G	PA2895	Hypothetical	Hypothetical, unclassified, unknown	Protease
102	43T7K	PA3173		Probable short-chain dehydrogenase	Swarming
107	G24T4	PA3001		Probable phosphate dehydrogenase	*Drosophila*
108	36T24G	PA3498		Probable oxido reductase	Protease
110	12T3K	PA5312		Probable aldehyde dehydrogenase	Pyocyanine
111	42T19T	PA5327		Probable oxido reductase	Twitching
113	15T23G	PA0041		Probable hemagglutinin	Swarming
114	28T21G	PA0584	*cca*	tRNA nucleotidyl transferase	Twitching, Drosophila
120	G20T2	PA0090		Probable ClpA/B-type chaperone	Swarming
127	96T13G	PA3876	*narK2*	Nitrite extrusion protein 2	Motility
128	11T16T	PA4887		Probable MFS transporter	Pyocyanine (++)
130	G94T2	PA0073		Probable ABC transporter	Swarming

131	76T14G	PA0151		Probable TonB-dependent receptor	Biofilm
133	G13T12	PA1863	*modA*	Molybdate-binding precursor	Swarming, twitching motility
137	35T12K	PA1157		Probable two-component regulator	Pyocyanine (++)

Abbreviation: C, conserved. Numbers in the left margin indicate STM clone numbers in Table 2 and in Table3.

Table 4. Bacterial strains and plasmids used in this work.

Bacterial strains	Relevant characteristics	Reference/Source
E. coli		
DH5α	*F-f80 ΔlacZΔM15 endA1 recA1 hsdR17 (r^-km^+k) supe44 thi-1 l-gyr A96 relA1 Δ(lacZYA-argF) U169*	Miller and Mekalanos, 1988
SM17λpir	*F^-araD Δ(lac pro) argE(Am) recA56 rif^r nalA,*	Taylor *et al*., 1989
P. aeruginosa		
PAO1 and PA14	Wild-type prototrophic laboratory strains	Holloway *et al.,* 1979
PAO909	*pur*A, *pur-67, E79 tv-2*, transduction of PAO910, used as negative control *in vivo*	*Pseudomonas* Genetic Center
PABS1	Derivative of PAO1, Δ *lipA*Δ *lipH* , defective in lipase	Wilhelm, S. *et al.,* 1999
	PAO1::mTn*5pil*, twitching motility mutant	Beatson *et al*., 2002
	PA14 *sad-36* (flgK), biofilm mutant	O'Toole and Kolter, 1998
Plasmids		
pTZ18R	pUC18, polylinker *lacZ*, lac^{iq} selection, Amp^r	
pUTminiTn*5*Km2	R6K-based suicide delivery plasmid, Km^r	De Lorenzo *et al*., 1990;
pUTminiTn*5*Tc	R6K-based suicide delivery plasmid, Tc^r	Herrero *et al.,* 1990
pUTminiTn*5*TcGFP	R6K-based suicide delivery plasmid, Tc^r, GFP	Matthyse *et al*., 1996

Table 5. Nucleotide sequences of signature tags used in multiplex PCR-based STM.

Tag number	Nucleotide sequence
1	GTACCGCGCTTAAACGTTCAG
2	GTACCGCGCTTAAATAGCCTG
3	GTACCGCGCTTAAAAGTCTCG
4	GTACCGCGCTTAATAACGTGG
5	GTACCGCGCTTAAACTGGTAG
6	GTACCGCGCTTAAGCATGTTG
7	GTACCGCGCTTAATGTAACCG
8	GTACCGCGCTTAAAATCTCGG
9	GTACCGCGCTTAATAGGCAAG
10	GTACCGCGCTTAACAATCGTG
11	GTACCGCGCTTAATCAAGACG
12	GTACCGCGCTTAACTAGTAGG
13	CTTGCGGCGTATTACGTTCAG
14	CTTGCGGCGTATTATAGCCTG
15	CTTGCGGCGTATTAAGTCTCG
16	CTTGCGGCGTATTTAACGTGG
17	CTTGCGGCGTATTACTGGTAG
18	CTTGCGGCGTATTGCATGTTG

19	CTTGCGGCGTATTTGTAACCG
20	CTTGCGGCGTATTAATCTCGG
21	CTTGCGGCGTATTTAGGCAAG
22	CTTGCGGCGTATTCAATCGTG
23	CTTGCGGCGTATTTCAAGACG
24	CTTGCGGCGTATTCTAGTAGG

Each 21-mers has a T_m of 64°C and permits PCR amplification in one step when the 3 primer combinations are used for multiplex screening. Two sets of consensus 5'-ends comprising the first 13 nucleotides have higher ΔGs for optimizing PCR. Twelve variable 3'-ends define tag specificity and allow amplification of specific DNA fragments. The set of 24 21-mers representing the complementary DNA strand in each tag are not represented and can be deduced from the sequences present.

Figure 1

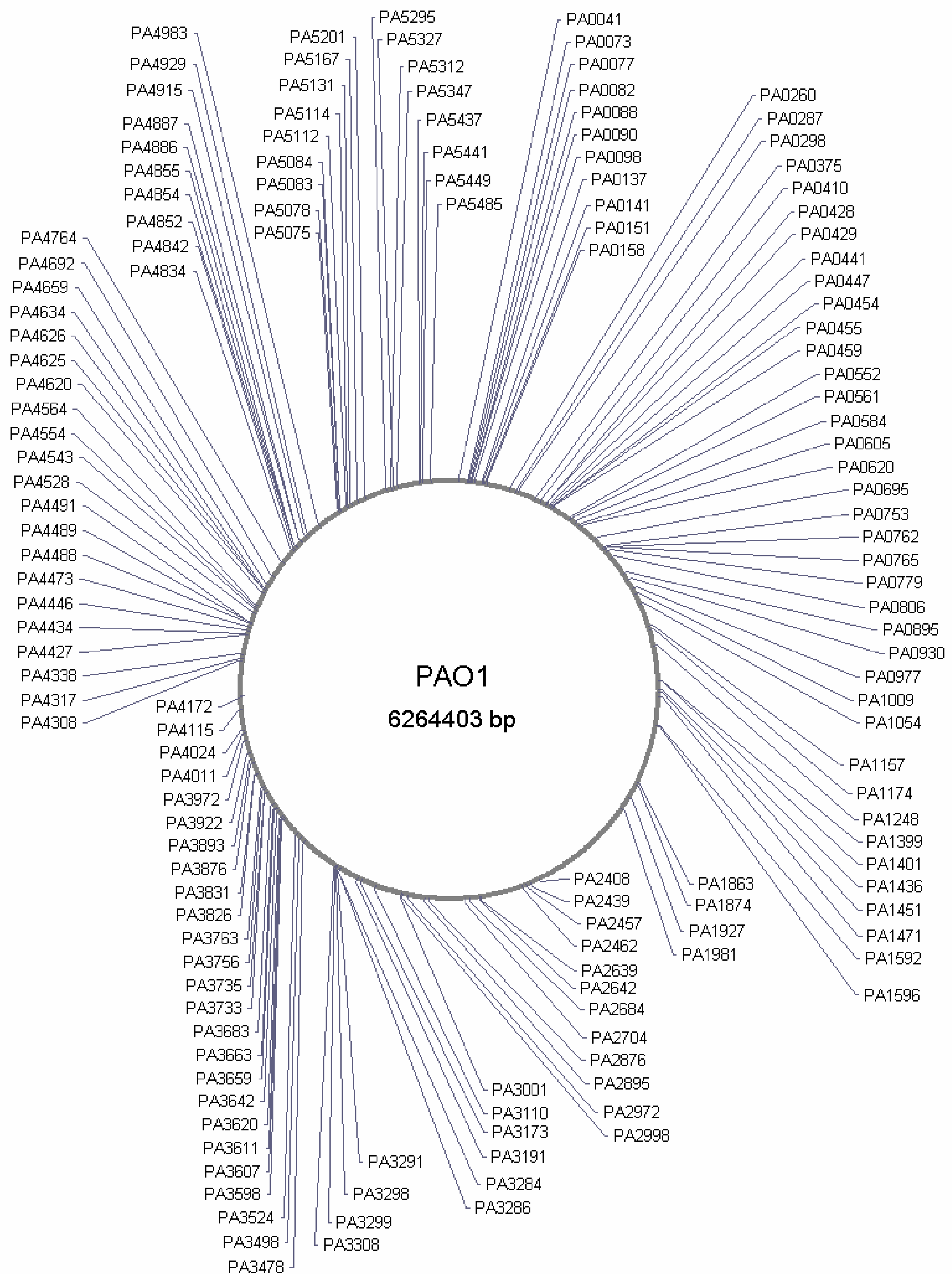


Figure 2

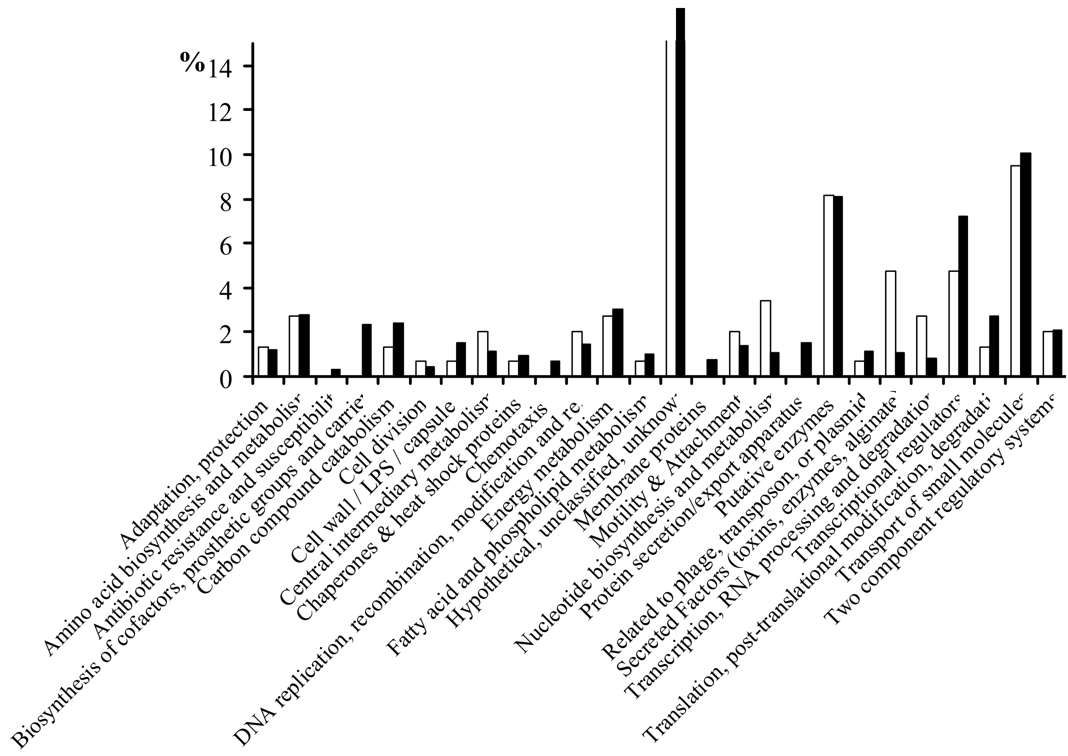


Figure 3

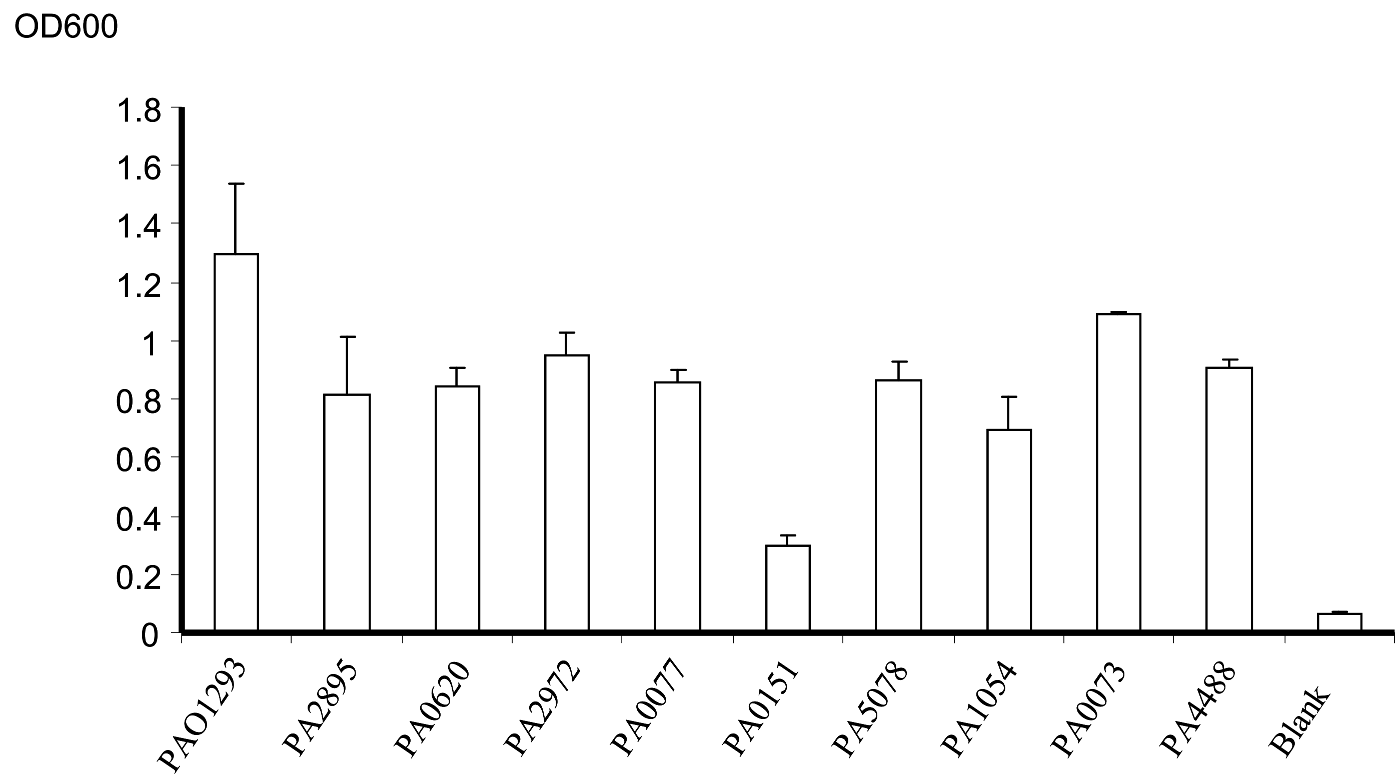


Figure 4

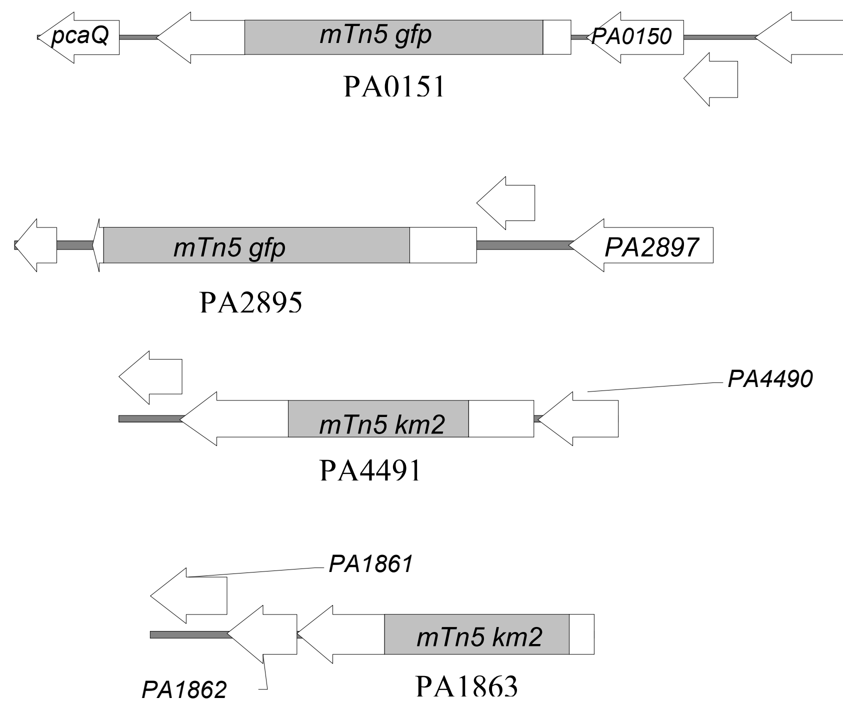
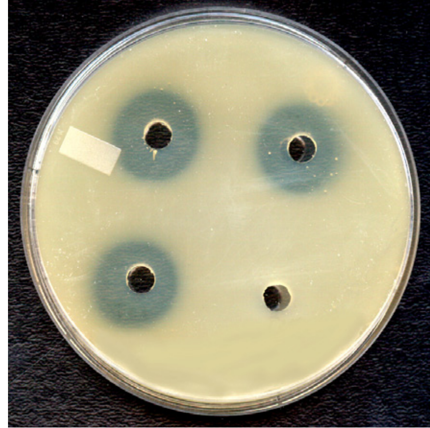


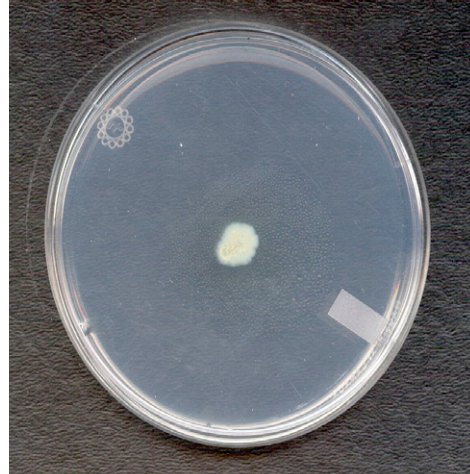
Figure 5

A



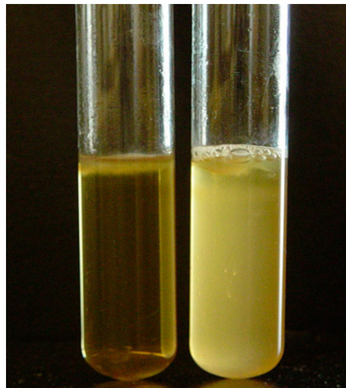
B

C



D

E



Chapitre 4

Génomique fonctionnelle d'un facteur sigma essentiel *in vivo* régulant les protéases extracytoplasmiques de *Pseudomonas aeruginosa*

Ce troisième et dernier chapitre est un article de recherche soumis pour évaluation à la revue *Molecular Microbiology*. Il regroupe essentiellement tous les résultats expérimentaux obtenus suite à la caractérisation biochimique et moléculaire de PA2895, un gène essentiel *in vivo*. Il a été démontré que le mutant STM2895 est incapable de dégrader la caséine. Cependant, nous nous attardons ici à démontrer que le défaut se situe plutôt au niveau du repliement d'au moins deux des quatre protéases extracellulaires de *P. aeruginosa*, soit les élastases LasA et LasB. Par des analyses génomiques et bioinformatiques, il est suggéré que PA2895 agirait comme senseur périplasmique régulant de façon négative l'activité transcriptionnelle de PA2896, un facteur sigma-70 de la sous-famille des ECF. Des analyses en transcriptome utilisant la technologie des puces à ADN d'Affymetrix ont permis de démontrer un lien étroit entre la régulation transcriptionnelle dépendante de PA2895 et PA2896 et le métabolisme du fer. Tous les travaux ainsi que la rédaction de ce manuscrit ont été réalisés par moi-même, avec l'aide technique et scientifique de nos collaborateurs du VCU et de l'UO.

Functional genomics of an essential *in vivo* sigma factor regulating extracytoplasmic proteases from *Pseudomonas aeruginosa*

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Summary

By secreting a large arsenal of virulence factors, *P. aeruginosa* causes lethal respiratory failure in CF patients. To understand host/bacterium interactions, we constructed a library of *P. aeruginosa* (PAO1) mutants using PCR-Based Signature-Tagged Mutagenesis. The mutant collection was screened in the rat model of chronic lung infection and identified 148 essential *in vivo* genes. A mutant of the PA2895 gene (STM2895), frequently recovered by the STM screening, was primarily identified by its exoproteases defect. Biochemical investigations using elastin congo-red, staphylolytic assay and poly-L-lysine degradation assays revealed that STM2895 was affected in LasA, LasB and lysine proteases. Immunoblots of STM2895 showed that LasB was present and correctly refolded and inactive whereas LasA accumulates in a processed form. A deletion Gm^R mutant of PA2896 was constructed and used with STM2895 in microarray experiments. Compared to wild-type PAO1, 128 genes (64 up and 64 down) for STM2895 and 138 genes (59 up and 79 down) for ΔPA2896::Gm were differentially regulated at ≥ 5 fold. Results from both STM2895 and ΔPA2896::Gm, were comparable in 76% of cases for upregulation and 59% for downregulation. Of significance is the number of operons or gene clusters involved in extracellular functions and genes associated to virulence (type III secretion pathway, exoenzymes, secondary metabolites and siderophores production). For repressed genes, 4 sigma-70 and PvdS (-24 fold) were highlighted as well as several genes identified with microarray data monitoring iron metabolism. In the STM2895 background, PA2896 was upregulated more than 9 fold confirming the negative regulation hypothesis and suggesting a feedback loop regulation. Competitive index (CI) confirmed the attenuation *in vivo* of STM2895 and a deleted mutant ΔPA2895::Gm had CI values of 0.074 and 0.116, respectively. We conclude that PA2895 is a negative regulator of PA2896, a putative ECF sigma-70 factor, and plays a major role in virulence.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen commonly found in association with immunocompromised hosts such as those with AIDS or burns and it also colonizes cystic fibrosis airways. *P. aeruginosa* is one of the leading causes of hospital-acquired nosocomial infections and demonstrates a high level of resistance to most classes of antibiotics (Banerjee and Stableforth, 2000). The production of exoproteases, lipases, phospholipases, exotoxines, motility apparatus, hydrogen cyanide, siderophores and the ability to produce exopolysaccharides such as alginate involved in biofilm formation represents an impressive arsenal used by the bacterium to bypass host defences (Lyczak *et al.*, 2000). Genomic analysis and annotation of the entire 6.3 Mb genome sequence of PAO1 revealed a repertoire of more than 550 transcriptional regulators (Stover *et al.*, 2000). Hence, more than 9 % of the large genome was predicted to encode transcriptional regulators and two-component environmental sensors. When compared to other microbial sequenced genomes, *P. aeruginosa* encodes 61 AraC, 115 LysR and 19 ECF (extracytoplasmic function) sigma-70 regulator-type families (Stover *et al.*, 2000).

Sigma factors are essential components of the RNA polymerase complex and determine promoter transcription specificity. Alternative sigma factors of the ECF-type respond to environmental changes and stimuli (Helmann, 2002; Lonetto *et al.*, 1994). In most cases, the activity of ECF sigma factors is modulated via one or more negative regulators including a cognate inner-membrane anti-sigma. These inner-membrane proteins are presumed to act as sensors or signalling molecules responding to environnemental changes (Missiakas and Raina, 1998). ECF sigma factors were shown to control responses to a variety of stresses associated with cell wall biosynthesis and protein folding. *P. aeruginosa* contains a locus homologous to the *E. coli* stress response regulator *rpoE rseABC* gene cluster, called the *algU mucABC* locus. AlgU controls the expression of the alginate biosynthesis pathway which confers the mucoid phenotype to *P. aeruginosa* cells (Rouviere *et al.*, 1995; Yu *et al.*, 1995). Recently, ECF sigma factors have been shown to be involved in bacterial pathogenesis and specifically in siderophore synthesis and uptake in *P. aeruginosa* (Bashyam and Hasnain, 2004). The importance of iron uptake and

metabolism in maintenance of chronic lung infections was shown to be mediated by PvdS and a number of homologues, all members of ECF subfamily (Visca *et al.*, 2002).

A PCR-Based Signature-tagged mutagenesis (PCR-STM) was used to generate a large *P. aeruginosa* strain PAO1 library of 7968 mutants to identify essential genes *in vivo* (Lehoux *et al.*, 2004). Using a PCR-based approach (Lehoux and Levesque, 2002), the complete library was systematically screened in the rat model of chronic lung infection and 148 genes were shown to be defective for *in vivo* maintenance. STM2895 which contains an insertional mutation in the functional gene PA2895 was previously identified as attenuated *in vivo*. Phenotypic analysis highlighted a defect in exoprotease production (Potvin *et al.*, 2003). A query search of the Pseudomonas database (http://www.pseudomonas.com) and annotation data predicted no putative function for PA2895. However, genomic analysis suggested that PA2895 is co-transcribed with a putative ECF sigma factor PA2896, sharing 31% identity with the FecI protein from *E. coli* (Braun *et al.*, 2003). The transcription of FecIR itself was shown to be dependant on the Fur protein and transcription is initiated by iron-limiting conditions (Braun, 1997). The aim of this paper is to define the role of the PA2895-PA2896 operon in the *in vivo* maintenance of *P. aeruginosa*. We focused our efforts on understanding the link between the exoprotease defect of STM2895 by western blot analysis and biochemical evaluation of LasA and LasB activity and its global impact on the transcriptional profiling patterns by the GeneChip technology. The competitive index was used to evaluate maintenance of STM2895 and PA2896 deletion mutants and complementation *in trans* in the rat chronic lung infection model.

Material and Methods

Bacterial strains and media

Bacterial strains used in this study are described in Table 1. *P. aeruginosa* and *E. coli* strains were routinely cultured in Luria-Bertani (LB) broth/agar at 37°C with antibiotics as needed. For some experiments, special culture media and conditions were used, and these are described further below. Antibiotics (Sigma) were used at the following concentrations: ampicillin, 100 µg ml^{-1} for *E. coli;* carbenicillin 500 µg ml^{-1} for *P. aeruginosa*; gentamicin 15 µg ml^{-1} for *E. coli* and 50 µg ml^{-1} for *P. aeruginosa*; tetracycline 10 µg ml^{-1} for *E. coli* and 35 µg ml^{-1} for *P. aeruginosa*. All solid and liquid media were purchased from Difco laboratories.

Identification of the mini-Tn*5*-Tc insertion points

Chromosomal DNA from STM2895 was extracted and purified using DNeasy tissue kit (Qiagen). *PstI* digested genomic DNA (300 ng) was ligated into the *PstI* site of pTZ18R (80 ng) (Amersham Biosciences). The ligation mixture was purified using a Microcon-PCR filtration device (Millipore) and electroporated (Gene-Pulser, Bio-Rad) into *E. coli* DH10B ElectroMAX competent cells (Invitrogen). Transformants were selected by plating on LB agar supplemented with ampicillin 100 µg ml^{-1}. Ampicillin resistant clones were collected and plated on tetracycline 10 µg ml^{-1}. Sequencing of the mini-Tn*5*/chromosomal DNA junction was performed using an oligonucleotide complementary to the I-end region of Tn*5* (5'-AGA TCT GAT CAA GAG ACA G-3'). The results generated by sequence analysis were compared to the Pseudomonas databases (http://www.pseudomonas.com).

Bioinformatics analysis

Bioinformatics analyses were done using software available online from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and the GCG (Genetics Computer Group, Wisconsin Package Version 10.3, Accelrys) software package. The genomic DNA sequence of the PA2895-PA2896 operon and the probable gene function of *P. aeruginosa* were obtained by using the Pseudomonas Genome Project and the PseudoCAP web database (http://pseudomonas.com). The PA2895-PA2896 ORFs of *P.*

aeruginosa were analysed for similarities to other proteins by comparing entries in the GenBank non-redundant database using the BLAST network service of NCBI.

Chromosomal DNA extraction (Southern hybridization)

Chromosomal DNA was extracted from *Pseudomonas* STM mutant strains using a modified version of this protocol (Meade *et al.*, 1982). Briefly, cells were grew overnight at 37°C in LB broth and subcultured at a 1/20 dilution into 10 ml fresh LB broth and incubated for 3 to 4 h at 37°C with aeration. Cells were harvested by centrifugation for 15 min at 3500 *g* and 4°C. The pellet was then washed twice in 10 ml cold saline and centrifuged as above. Cells were suspended in 10 ml cold TE 10 (10 mM Tris-HCl pH 7.6, 10 mM EDTA). One ml of lysozyme (Sigma) solution 2 mg ml^{-1} in TE 10 (freshly prepared) was added to the cell suspension and incubated 15 min at 37°C. Proteins were removed by adding 2 ml of Pronase (Roche Diagnostics) 5 mg ml^{-1} in TE 10 containing 10% N-lauryl sarcosine, and incubated at 37°C for 1 h. The suspension was extracted at least twice with 15 ml phenol/chloroform (1:1) and mixed thoroughly by inversion. Phases were separated by centrifugation at 5000 *g* for 10 min. The aqueous phase was recovered and extracted twice with 15 ml chloroform and phases were separated as previously described. For every 10 ml of aqueous layer, 1.2 ml of 3M sodium acetate pH 5.2 was added. DNA was precipitated by adding 2 volumes of isopropanol at room temperature, mixed by inversion and stored at -80°C overnight. DNA was pellet by centrifugation at 5000 *g* for 10 min. The DNA pellet was then washed twice with cold ethanol 70% and air dried. DNA was dissolved in 1 ml of 10 mM tris-HCl pH 7.6, 1 mM EDTA. DNA concentrations were determined by UV absorption at 260 nm.

Southern Blot hybridization

All reagents for Southern hybridization and labelling were purchased from Roche Diagnostics and used as recommended by the manufacturer. The probe selected corresponds to the GFP region of mini-Tn*5* GFP used in the screening of the STM library (Lehoux *et al.*, 2004). The 820 bp DNA fragment was labelled with the dioxygenin-11-dUTP using the PCR DIG Probe Synthesis kit with primers tag24 (5'-CTT GCG GCG TAT TCT AGT AGG-3') and gfpR2 (5'-ATC CAT GCC ATG TGT AAT CCC-3').

Amplification conditions were: hot start at 95°C for 15 min followed by a touchdown step 22 cycles (95°C, 1 min; 70-60°C, 1 min decreased by 1°C every 2 cycles; 72°C, 1 min), 10 cycles (95°C, 1 min; 60°C, 1 min; 72°C, 1 min) and a final extension step at 72°C for 7 min. DNA fragments obtained from 2 µg of *PstI* (New England Biolabs) totally digested genomic DNA from PAO1 and STM2895 were separated on 1 % (w/v) agarose gel, and blotted onto a positively charged nylon membrane (Roche Diagnostics). Before hybridization, the agarose gel was treated as follows: depurination for 20 min (250 mM HCl), denaturation 2 X 15 min (0.5 M NaOH, 1.5 M NaCl), 2 X 15 min neutralization (0.5 M Tris-HCl pH 7.5, 1.5 M NaCl), equilibration 10 min (20X SSC: 300 mM sodium citrate, pH 7.0, 3 M NaCl) and fixing (UV cross-linking, 3 min). The membrane was prehybridized with the DIG easy Hyb for 30 min at 37ºC, incubated with the GFP probe (100 ng) for 18 h at 42ºC and washed with 2X SSC and then with 10X SSC. The membrane was blocked with the DIG wash and Block buffer set and revealed using the Anti-Dioxigenin-AP Fab fragments (dilution 1:5000). CSPD chemiluminescent substrate was diluted in the detection buffer (1:100) and used to reveal the hybridization.

Protease assays

For determination of exoprotease activity, *P. aeruginosa* strains were cultured in 2 ml of LB broth in 13 mm test tubes without agitation at 37°C for 48 h (Laux *et al.*, 2002). Cell-free supernatant samples were collected by centrifugation for 15 min at 7000 *g*. and filtered on a 0.45 µm filtration device (Millipore). Aliquots of 100 µl were used to inoculate a brain-heart infusion agar plate containing 1% (w/v) skim milk (Difco Laboratories). Aliquots were dropped into wells and proteolysis zones were visualized after an overnight incubation at 37°C. Elastolysis activity (LasB) was determined with the elastin congo-red assay according to a modification of a previously reported procedure (Ohman *et al.*, 1980). To 15 ml polypropylene screw-cap tubes (Falcon), 10 mg of elastin congo-red (Sigma) and 2 ml of reaction buffer (100 mM Tris-maleate buffer, pH 7.0, 1 mM $CaCl_2$) was added. Supernatant samples were prepared as described above and 1 ml was added to the reaction mixture. Tubes were incubated horizontally overnight at 37°C on a rotary plate at 250 r.p.m. The remaining substrate was removed by centrifugation at 1500 *g* for 5 min and absorbance was read at 495 nm. Elastase activity was expressed as ΔOD at 495 nm and

normalized with bacterial densities present in primary static cultures (OD 600 nm). Elastase A (LasA) activity was measured using a staphylolytic assay (Park and Galloway, 1995). *Staphylococcus aureus* strain ATCC 25923 was cultured into 25 ml tryptic soy broth overnight at 37°C, cells were centrifuged and resuspended into 12 ml of DE buffer (25 mM diethanolamine buffer, pH 9.5). Cells were heat-killed by boiling at 100°C and diluted in DE buffer to a final OD_{595} of 1.5. In 13 X 100 mm glass tubes, the heat-killed *Staphylococcus* suspension (1 ml) was incubated in the presence of aliquots of 300 µl of *Pseudomonas* supernatants and 700 µl of DE buffer at 37°C for 60 min. Optical densities were measured at 595 nm. Elastase A activity was observed by the decreasing of absorbance values compared to a negative control replacing supernatant by LB broth (ΔOD_{595}). Optical values were normalized to the starting amount of bacteria in primary static culture (OD 600 nm).

Plasmid constructions

All plasmids in this study are listed in Table 1 and plasmid DNAs were purified by using the Qiaprep spin miniprep kit or the Qiafilter plasmid midi kit (Qiagen). Standard recombinant DNA manipulations techniques were used (Sambrook and Russell, 2001). Restriction and modification enzymes were purchased from New England Biolabs and used as recommended by the manufacturer. PCR reactions were performed using a Hotstart Taq Kit (Qiagen). Oligonuleotides synthesized by Invitrogen were used for sequencing and PCR. The starting PAO1 chromosomal DNA template used for PCR amplifications was purified by the procedure described above. Plasmid pMON3401, used for complementation *in trans*, was constructed by cloning a 1037 bp PCR fragment encoding the PA2895 gene using the primers CO2895sense (5'-CAG TTC TCT AGA CCG AGA TCG CCA CGC TCA CCC AGT C- 3') and CO2895Asense (5'-CAG TAC GCG CTC TTG CGG CAA TGC TGA CGG CAG ACT G-3') and was ligated into the pUCP19 *Pseudomonas* shuttle vector (West *et al.*, 1994). PCR conditions used were hot start at 95°C for 15 min followed by a touchdown step 22 cycles (95°C, 1 min; 65-55°C, 1 min decreased by 1°C every 2 cycles; 72°C, 1 min), 10 cycles (95°C, 1 min; 55°C, 1 min; 72°C, 1 min) and a final extension step at 72°C for 7 min. PCR final mix was supplemented with 5% (v/v) DMSO. The plasmid was introduced into *E. coli* DH10B ElectroMAX competent cells (Invitrogen)

and subsequently into *P. aeruginosa* STM2895 by electroporation (Enderle and Farwell, 1998). Plasmid pMON3429, used as starting material for allelic exchange, was constructed by cloning the 728 bp fragment encoding the gene PA2896 and generated by PCR with primers 2896Sense (5'-CCT AGA GAG CTC ATG CGT GCC GCG AAG GAT G-3') and 2896ASense (5'-GGA ACC TCT AGA CGG AAA TGC GCC AGC ATC-3') using *SacI*/*XbaI* restriction sites of pUCP19. PCR conditions were a hot start at 95°C for 15 min followed by a touchdown step 22 cycles (95°C, 1 min; 55-45°C, 1 min decreased by 1°C every 2 cycles; 72°C, 1 min), 10 cycles (95°C, 1 min; 45°C, 1 min; 72°C, 1 min) and a final extension step at 72°C for 7 min. The PCR final mix was supplemented with 2.5% (v/v) DMSO. *P. aeruginosa* strains with deletions of PA2895 and PA2896 were generated using allelic replacement and the levan-sucrase lethality phenotype (5% sucrose) with the counter-selectable *Bacillus subtilis* SacB marker (Hoang *et al.*, 1998). Plasmid pMON3401 containing PA2895 was deleted of a 604 bp *AvaI/BsmI* DNA internal gene fragment, the remaining vector portion was gel purified using Perfectprep Gel Cleanup Kit (Eppendorf), blunt-ended and ligated with the 1078 bp Gm-FRT cassette. The 1698 bp *SfiI/HindIII* DNA fragment was excised and cloned blunt-end into the *SmaI* site of pEX18Tc (AF047519) to generate pMON3440. For PA2896, an internal *BsmI/ClaI* 324 bp DNA fragment was deleted and a blunt-end ligation of the Gm-FRT cassette was inserted giving pMON3429. The 1669 bp *SfiI/HindIII* DNA fragment was cloned blunt-end into *SmaI* site of pEX18Tc to generate pMON3441. Plasmids were introduced into the *E. coli* donor strain SM10 and transfered by conjugation on 0.45 µm filter on BHI agar plates into the recipient strain *P. aeruginosa* PAO1. Transconjugants were selected on Pseudomonas Isolation Agar (PIA) supplemented with tetracycline 35 µg ml^{-1}. Tetracycline resistant clones were collected and plated on PIA + gentamicin 150 µg ml^{-1} supplemented with 5% (w/v) sucrose to select for double events of homologous recombination. The gentamicin and sucrose resistant clones were purified by streaking on PIA + gentamicin 150 µg ml^{-1} supplemented with 5% (w/v) sucrose and screened for tetracycline sensitivity. Chromosomal deletions mutant strains were confirmed by PCR using complementation primers (see above) and DNA obtained from boiled bacteria.

Western Blot analysis

Immunoblots were done as described by (Olson and Ohman, 1992). Briefly, supernatants from 48 h culture were concentrated 10 fold with the Microcon YM-10 (Millipore) and an aliquot of 20 µl was mixed with 10 µl of concentrated sample buffer (65 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 0.5% (v/v) β-mercaptoethanol). Samples were boiled 3-5 min and loaded adjacent to a pre-stained protein standard (New England Biolabs) and resolved on 15% (w/v) SDS-PAGE (Laemmli, 1970) or 4-20 % (w/v) gradient Pre Cast acrylamide gels (Gradipore). Proteins were blotted onto a PVDF membrane (Bio-Rad). Polyclonal rabbit anti-LasB 1:300 000 and polyclonal rabbit anti-LasA 1:2000 were used as primary antibodies; and donkey anti-rabbit HRP-coupled used as secondary. Membranes were washed using 1X PBS + 0.5% (v/v) Tween 20 three times for 5 min at room temperature between antibody incubations and blocked with ECL Donkey Anti-rabbit IgG Peroxidase-linked Kit (Amersham Biosciences). The detection was performed with the ECL Advance Western Blotting Detection Kit (Amersham Biosciences) as recommended by the manufacturer.

Sampling, RNA extraction and transcriptional profiling

For GeneChip analysis, *P. aeruginosa* strains were grown in LB broth. One hundred microlitre from an overnight test tube static culture was used to inoculate 100 ml LB broth incubated in a 0.5 L Erlenmeyer flask at 37° C with aeration. Cells from late-logarithmic phase cultures (OD_{600} = 0.8) were collected and RNA degradation was minimized by adding 12.5 ml of ice-cold 5% (v/v) phenol in absolute ethanol (pH < 7.0). RNA was extracted and purified using a previously reported procedure (Bowtell and Sambrook, 2003). DNA contamination of purified RNA was monitored using PCR for amplification of the *rplU* gene with the primers rplU-for (5'-CGC AGT GAT TGT TAC CGG TG-3') and rplU-rev (5'-AGG CCT GAA TGC CGG TGA TC-3'). RNA integrity was monitored by agarose gel electrophoresis of glyoxylated samples. Preparation of labeled cDNA and processing of the *P. aeruginosa* GeneChip arrays was performed as described previously (Schuster *et al.*, 2003). Washing, staining, and scanning of the GeneChips were performed by the University of Iowa DNA core facility using an Affymetrix fluidics station. GeneChip data were analyzed using GeneChip Operating Software (Affymetrix).

***In vivo* competitive index (CI)**

The chronic rat lung model of infection previously described (Cash *et al.*, 1979), was used to confirm the *in vivo* competitive index (CI) and the previously observed defect in *in vivo* maintenance for STM2895 (Potvin *et al.*, 2003). Three male Sprague-Dawley rats (300-350 g) (Charles River, Canada) were utilized for determination of the CI for each mutant: STM2895, PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm mixed with the *P. aeruginosa* wild-type strain PAO1 harbouring pUCP19 (that conferred carbenicillin resistance) (see Table 1). All rats were inoculated intratracheally with 5 X 10^3 CFUs for the wild-type and mutant and mutant strains giving a total dose of 1 X 10^4 *P. aeruginosa* CFU embedded in agarose beads prepared as described (van Heeckeren and Schluchter, 2002). Inoculums were injected into rat lungs using a 1 ml tuberculin syringe containing 100 µl of bead slurry. Animals were sacrificed and lungs were collected at 7 days post-infection. Lungs were homogenized with Polytron and total CFUs were determined by plating serial dilutions on PIA. Mutant and wild-type CFUs were determined by plating the dilutions on selective media MHA + gentamicin 15 µg ml^{-1} for PAO1 ΔPA2895::Gm, PAO1 ΔPA2896::Gm, MHA + tetracycline 15 µg ml^{-1} for STM2895 and MHA + carbenicillin 500 µg ml^{-1} for PAO1 + pUCP19. CI was expressed as the number of CFUs from the mutant strain divided by the number of CFUs from the wild-type strain (Lehoux *et al.*, 2000).

Results and Discussion

Genomic organization and features of PA2895-PA2896

The genomic organization of the PA2895 operon is presented in Fig. 1A and would encompass at least 2 genes. The transcription of PA2895 and PA2896 is polycistronic and there is a clear evidence for overlapping of the initiation start and stop translation codons for both PA2895 and PA2896 ORFs. Moreover, a Shine-Dalgarno sequence was found upstream of the translation start site (Fig. 1B). Bioinformatics analysis of the deduced polypeptides from PA2895 and PA2896 showed interesting features. The BLASTP program that uses update databases from the NCBI (http://www.ncbi.nlm.nih.gov) found only one close homologue in the recently released unannotated genome of *P. aeruginosa* strain UCBPP-PA14 but no predicted function. According to Pseudomonas.com databases, PA2895 encodes a predicted 23 amino acid length transmembrane domain between amino acids 67 and 89. PA2896 is predicted to encode a putative transcriptional regulator of the sigma-70 protein with an ECF subfamily signature (PS01063) (Fig. 1A). The PA2896 putative peptide has homology with a high number of uncharacterized ECF-type sigma factor homologues, including two conserved regions r2 (pfam04542) and r4 (pfam04545). These two regions are involved in the recognition of the -10 and -35 promoter sequences. PA2896 also has a 31% identity with the FecI protein from *E. coli* K-12 (Braun *et al.*, 2003). Significant identities (48%) to AlgU and SigX of *P. aeruginosa* were also noted. The genomic organization of PA2895-PA2896 is in agreement with the basic characteristics normally attributed to ECF sigma factors-based regulation (Helmann, 2002). Briefly, PA2896 is co-transcribed with PA2895, a putative transmembrane sensor which could possess an anti-sigma function, and a demonstrated influence on extracytoplasmic functions. We hypothesized that PA2895 could be the anti-sigma factor involved in the

transducing of a specific, as yet to be identified, signal from the periplasm activating PA2896-dependent transcription, and causing the exoprotease defects described here.

STM2895 is defective in P. aeruginosa major virulence factors

PA2895 was shown to be one of 214 essential *in vivo* genes identified previously by the screening of a large 7968 STM mutants in the rat lung model of chronic infection (Potvin *et al.*, 2003). The mini-Tn*5*-Tc GFP insertion of STM2895 was determined by nucleotide sequencing of the junction between the chromosomal and the transposon DNAs of a *PstI* DNA fragment cloned in pTZ18R which confers tetracycline resistance using as a primer an oligonucleotide encoding the I-end (see material and methods section). Sequencing revealed that the transposon was inserted at the first nucleotide of codon 218. The disrupted 254 amino acids length PA2895 protein is not translated in STM2895. (Fig. 2A). Analysis of insertions in the 214 STM clones confirmed the PA2895 had 11 different insertions and was the most frequently isolated. As shown in Fig. 2B, Southern blot hybridization was performed and indicated the presence of one copy of the transposon in a 3.7 kb *PstI* chromosomal DNA fragment of STM2895 (Fig. 2B).

Using a qualitative skim milk plate degradation assay, *P. aeruginosa* STM2895 was shown to be defective in production of exoproteases. As shown in Fig. 3, the wild-type *P. aeruginosa* strain PAO1 generated a halo of casein degradation that can be compared to those produced by the complemented strain *in trans* with a functional copy of PA2895 STM2895 + pMON3401 and the PDO801 mutant. β-casein degradation is known to be the preferential substrate of the alkaline protease and mainly the LasB elastase (Caballero *et al.*, 2001). The absence of casein degradation by STM2895 confirmed that the LasB elastase is inactive; similar results were obtained with the PDO240-1 strain. Thus, one of the major secreted proteases of *P. aeruginosa*, LasB elastase, is inactive in the STM2895 mutant. To confirm these results and to analyse exoprotease defect specificity, a combination of assays including the elastin congo-red (ECR), staphylolytic (SA) and poly-L-lysine degradation assays (PLD) were performed with the same strain collection. Preliminary experiments using culture supernatants in anaerobic growth conditions (static 2-days culture) were tested in ECR and SA assays and confirmed results obtained with plate assay. As shown in Table 2, the level of staphylolytic elastase A activity detected for

STM2895, for the PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm is virtually zero and comparable to the PDO801 control strain. The activity of LasA elastase is fully recovered in the STM2895 strain harbouring the pMON3401 plasmid *in trans* expressing the PA2895 protein. The PDO240-1 strain which is deficient in the production of LasB elastase has reduced LasA activity since LasB was shown to be important in the processing of LasA (Kessler *et al.*, 1998). LasB elastase is known to be the most abundant protease secreted by *P. aeruginosa* (McIver *et al.*, 2004) and is one of the major virulence factors (Woods *et al.*, 1982). An Elastin congo-red substrate assay specific for LasB was used to determine the influence on the activity of LasB elastase in a PA2895-PA2896 mutant background. As shown in Table 3, STM2895, PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm strains produced low amounts of elastinolytic activity due to LasB, when compared to wild-type PAO1. As LasA and LasB enzymes are known to be induced in the late log phase of growth via the induction of quorum-sensing, we wanted to verify if the protease defect associated to LasA and LasB was dependant on a specific growth point when compared to wild-type expression. Fig. 4 shows growth curves realized in aerobic conditions with a collection of the four most relevant strains. His clearly confirmed that none of these strains were altered in their ability to grow when compared to wild-type PAO1. Using supernatant samples of these cultures, ECR and SA assays were performed to verify the exact point of elastases induction via the PAO1 strain and the associate protease defect of the STM2895 mutant strain. As depicted by Fig. 5 and 6, LasA and LasB activities are detectable around 1-1.2 OD 600 as predicted by the QS induction machinery in late log - early stationary phase. On the other hand, STM2895 produce virtually no more exoprotease activity over detectable elastinolytic activity throughout the growth spectrum and confirmed that the LasA and LasB defect is constitutive. Surprisingly, the complemented strain *in trans*, with the functional copy of PA2895, seems to accumulate expression delay of the proteolytic enzymes and recovered only a small amount of the lost activity. This phenomenon is likely due to the aerobic growth conditions as static culture showed the fully recovered phenotype.These observations proposed a noticible link between the expression of PA2895 and oxygen availability.

P. aeruginosa secretes two other exoproteases in its supernatant; the lysine-protease protease IV encoded by the *prpL* gene (Wilderman *et al.*, 2001) and an alkaline protease

(Morihara *et al.*, 1965). The PLD assay is specific for lysine proteases activity and was previously demonstrated to be able to degrade poly-L-lysine substrate (Caballero *et al.*, 2001). Fig. 8 clearly demonstrates that lysine proteases are affected in STM2895 strains. However, the PLD assay cannot discriminate between protease IV and alkaline protease activities. The presence of a lower spot in lanes 2 and 3 in Fig. 8 confirmed that the poly-L-lysine substrate is partly degraded, in contrast to the PAO1 wild-type strain (lane1, in Fig. 8). This defect can also complemented *in trans* in STM2895 using a functional copy of PA2895 expressed in the pMON3401 plasmid. These results suggest that LasA, LasB and one or perhaps both lysine exoproteases are inactive in the mutant strains of PA2895 and PA2896.

Synthesis and secretion of elastases is not altered in the STM2895 mutant

To determine the level of production of LasA and LasB elastases and confirm if they are inactive in culture supernatants of STM2895, PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm, we performed immunoblots to detect the presence and the integrity of these two exoprotease proteins. SDS-PAGE analysis demonstrated that the major exoprotease, LasB elastase, is present in a mature form of 33 kDa for the PAO1 wild-type strain, and is shown in lane 1 of Fig. 7A-B (Kessler *et al.*, 1998). When compared to other strains, LasB is also detected in a correctly processed form in mutant strains STM2895, PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm; and is totally absent in the PDO240-1 mutant strain used as a control. Moreover, separation of proteins secreted into the supernatant by SDS-PAGE revealed the presence of a protein of 20 kDa corresponding to LasA elastase that accumulated in the supernatants of STM2895, STM2895 + pUCP19, and both mutants ΔPA2895 and ΔPA2896. Immunoblots performed with anti-LasA and anti-LasB polyclonal antibodies (Fig. 7B and Fig. 7C) confirmed these observations. Fig. 7B shows that the PAO1 wild-type strain and the complemented strain STM2895 + pMON3401 produced a lower amount of LasA elastase when compared to STM2895, PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm strains. LasA is present in the supernatant of STM2895 and in strains with deletions in PA2895 or PA2896, but it accumulates in a processed form of 20 kDa and remains inactive. Western blot analysis using an anti-LasB antibody showed that LasB elastase is also present in culture supernatants of all strains tested, except for PDO240-1

Δ*lasB* strain (Fig. 7C). When compared to wild-type PAO1 and the complemented strain, STM2895 and the ΔPA2895 and ΔPA2896 mutants produced significantly less detectable LasB elastase. We conclude that LasA and LasB elastases are produced by the STM2895 mutant strain in a correctly processed form, but both remain inactive. It is known that these enzymes have complex periplasmic refolding steps, involving disulfide bonds formation (Braun *et al.*, 2001).

Transcription profiling of genes affected by PA2895-PA2896

To identify genes regulated by PA2895-PA2896 in *P. aeruginosa*, we used the complete genome transcriptional analysis GeneChip from Affymetrix and compared transcript profiles between the STM2895 mutant and an isogenic mutant of PA2896 with the transcription profile of the parent strain PAO1. For STM2895 and for PAO1 ΔPA2896::Gm, we noted changes in 128 genes (64 up and 64 down) and 138 genes (59 up and 79 down), 4 intergenic regions and 12 tRNA coding regions which were shown to be differentially regulated by ≥ 5 fold. When compared together, transcriptional data from both chips were similar. In fact, the mutation created in PA2896 presumably gave a polar effect for PA2895, aborting its transcription because of the insertion of the gentamicin resistance cassette upstream. Results for upregulational genes are summarized in Table 2. Results for downregulated genes are presented in Table 3. Indeed, there are 76% (53 on 70 different genes listed) of upregulated expressed genes common for both mutants and 59% (53 on 90 different genes listed) for downregulated genes. These percentages take into account only values above a 5 fold threshold, as presented in Tables 2 and 3. Genes regulated by PA2895 and PA2896 in both chips have comparable values in terms of fold change. In 26% of cases upregulation difference between fold change values is greater than 3. For downregulation, 42% of coregulated genes have a significant difference greater than 3 fold in both experiments.

The most interesting and significant data concerning induced genes by PA2895 and PA2896 is the number of operons, or gene clusters, involved in extracellular functions and genes associated with virulence. For instance, several regulated genes are members of the type III secretion system, phenazine, hydrogen cyanide and pyochelin biosynthesis, exoenzymes Y and S synthesis and elastase B; all of which are involved in virulence of *P.*

aeruginosa. Another gene cluster upregulated in STM2895 and PAO1 ΔPA2896::Gm mutants is PA3327-PA3336 including cytochrome P-450 which is probably involved in enzymatic detoxification of antibiotics (Desomer *et al.*, 1992; Munro and Lindsay, 1996). Also upregulated are two operons involved in transport of the small molecules PA2110-PA2114 which include a probable porin PA2113, a probable MFS transporter PA2114 and a group of probable ATP dependent ABC transporters PA3187-3190. Finally, an operon of uncharacterized function PA0492 to PA0496 was upregulated in both mutants tested.

The induced genes identified here also re-group genes that are not yet characterized as part of an operon and include three transcriptional regulators. The denitrification regulator Dnr protein and a probable transcriptional regulator PA1196 in both strains tested and PA2896 was found only in the STM2895 mutant. This would indicate a feedback loop regulation of the PA2895-PA2896 operon. This interesting result supports the hypothesis that PA2895 could act as an anti-sigma negative regulator on sigma-70 ECF PA2896. The high positive values of +55.7 for upregulation of PA1494 and +13.0 for PA4495 found only in STM2895 transcription profiling and not in PAO1 ΔPA2896::Gm suggested that these genes are regulated by PA2896.

The repressed genes for transcription profiling of STM2895 and ΔPA2896::Gm are listed in Table 3. The analysis of repressed genes controlled by the PA2895-PA2896 regulon revealed that the hierarchy of regulation involved at least 5 other sigma-70 factors of the ECF subfamily, such as PA0472, PA1300, PA3899 and PA4896 of uncharacterized function and the iron-regulator PvdS (PA2426). Moreover, PA0149, PA1912, PA2468 and PA3410 are also sigma-70 factors (ECF subfamily) that are downregulated, but less than 5 fold (data not shown). Other transcriptional regulators are significantly repressed such as *rsmA*, which is involved in post-transcriptional repression of secondary metabolites including exoproteases, hydrogen cyanide and phenazine compounds (Pessi *et al.*, 2001). Downregulation of the RsmA regulator would thus be linked to the induced response of secondary metabolites described above. Another transcriptional regulator identified as repressed is *pchR* (PA4227), an AraC-like regulatory protein, which is involved in the production of the pyochelin siderophore (Heinrichs and Poole, 1996). We also noted a strong repressive effect on *nir*, *nor* and *nar* genes by the PA2895-PA2896 mutations. These

genes are involved in the denitrification pathway of *P. aeruginosa* and have been shown to be regulated by Anr/Dnr proteins (Arai *et al.*, 1995) (Vollack *et al.*, 1998). The cytochrome o complex (*cyo* genes), the predominant terminal oxidase in the aerobic respiratory chain of *E. coli* and related bacteria such as *P. aeruginosa* grown under conditions of high aeration, are also repressed in the absence of PA2896 (Chepuri *et al.*, 1990). Other genes associated with oxygen transfer and detoxification are significantly repressed and include probable oxidoreductases (PA1137, PA4615), *hmgA* dioxygenase, superoxyde dismutase and the strongly repressed *fhp* gene flavohemoprotein which has been recently shown to protect against oxidative shock in *E. coli* (Justino *et al.*, 2005). Probably the most relevant finding revealed by the study of the PA2895-PA2896 regulon is the high number of genes that are shared with previously reported microarray experiments in relation to iron metabolism. Effectively, Palma *et al.*, 2003 and Ochsner *et al.*, 2002 published two exhaustive lists of genes that are differentially regulated in response to iron and iron starvation identified in bold in Tables 2 and 3. These results suggest that PA2895-PA2896 could control the response to iron at another level by downregulation of PvdS, one of the major iron regulators. This would have a major impact for *in vivo* maintenance. *P. aeruginosa* possesses an obligatory oxidative metabolism, the iron-based oxygen transport is highly used during growth in full aeration; this kind of metabolism is the most energetic but also the most toxic (Vasil and Ochsner, 1999). In fact, during oxygen-based respiration processes free-radicals are created and lead to protein damage (Hassett *et al.*, 1999). The absence of this kind of detoxification system is likely leading to changes in the redox potential of the periplasm, resulting in the elastases misfolding.

PA2895 is essential for in vivo maintenance

Competitive Index (CI) aimed to analyse the ability of a specific *P. aeruginosa* mutant strain to compete for maintenance in the rat lung. As noted in Table 6, STM2895 and PAO1 ΔPA2895::Gm strains are significantly attenuated when challenged with PAO1 parent strain. In fact, CI values (0,074 and 0,116) confirmed that mutations in the PA2895 gene cause a fatal reduction of approximately 1 log CFUs compared to the wild type PAO1 in the rat lung. We also tested the PAO1 ΔPA2896::Gm strain in the same experiments and surprisingly, CI value was greater than 1 (>1) indicating that this strain is able to maintain

chronic infection as PAO1 wild-type strain. We hypothesized above that the insertion of the gentamicin resistance cassette (1 kb) in the ΔPA2896::Gm strain could act as a polar effect on the transcription of PA2895 causing the same observable exoprotease defect and the comparable microarray results for the strain STM2895. But it is noticeable to remember that the PA2896 gene was not identified by our STM screening and by any other *in vivo* studies. This result suggests certainly an important implication of growth phase and culture conditions in the observation of the different phenotypes described on here the PA2895 knock-out strain and also likely suggest a multipartite regulation network.

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Figure legends

Fig. 1. Genetic organization of the *P. aeruginosa* PAO1 PA2896-PA2895 region under study. (A) Schematic depiction of the two ORFs represented by arrows and in-box summarizes of relevant genomic features from both genes. Gene and protein lengths, protein topology and putative function information were imported from pseudomonas.com databases. Homologues were found using BLASTP software from NCBI. (B) Genomic sequence analysis and proper translation showing the overlap of both ORFs on one codon (bold) and a putative Shine-Dalgarno sequence (underline) upstream of the translation start site of PA2895.

Fig. 2. Unique transposon insertion of STM2895. (A) Schematic representation of mini-Tn*5* gfp tag 24 inserted in gene PA2895. The mini-Tn*5* (light green) is represented by DNA insertion that disrupted PA2895 (blue). The horizontal bar represents the 3.7 kb pst I fragment visualized on Southern blot analysis (B) using the dig-labelled 820 pb gfp probe (in red). STM2895 DNA track 1, PAO1 DNA track 2, control unlabelled PCR product probe track 3.

Fig. 3. Exoproteases analysis of STM2895. Proteolytic activity was measured qualitatively from supernatant from different strains of *Pseudomonas aeruginosa* using BHI agar plates containing 1% skim milk. Supernatant samples were collected from 48 hour static cultures and 100µl was dropped onto previously removed agar plugs and proteolytic activity was visualized after incubation overnight at 37°C.

Fig. 4 Growth curves. Growing ability in aeration conditions of four relevant *Pseudomonas aeruginosa* strains was measured using optical densities (OD) at 600 nm following a time course. An aliquot from overnight cultures was used to inoculate tryptic soy broth (supplemented with appropriate antibiotics) to a starting OD of 0.01 from an overnight culture of these strains. Green curve: PAO1 wild-type, red curve: STM2895, black curve: STM2895+pUCP19, blue curve: STM2895+pMON3401.

Fig. 5. Elastase LasB activity. Elastin-Congo red degradation assays were performed on selected triplicate samples from growth curve experiments. LasB activity was quantitated by a single overnight optical density value at 495 nm using the cell-free supernatant from the collected samples and resulting from the degradation of elastin covalently linked to congo-red. Green bar: PAO1 wild-type, red bar: STM2895, black bar: STM2895+pUCP19, blue bar: STM2895+pMON3401.

Fig. 6. Elastase LasA activity. Staphylolytic assays were performed on selected triplicate samples from growth curve experiments. LasA activity was evaluated by a single 60 minute elapsed optical density value at 595 nm using the cell-free supernatant from the collected samples and resulting from the degradation of a heat-killed Staphylococcus aureus suspension. Green curve: PAO1 wild-type, red curve: STM2895, black curve: STM2895+pUCP19, blue curve: STM2895+pMON3401.

Fig. 7. Exoproteins analysis. (A) Culture supernatants were concentrated 10-fold using Amicon ym-10 column and samples were resolved on a 15% SDS-PAGE. (B) Proteins were blotted on a PVDF membrane and polyclonal rabbit anti-LasB 1:300 000 or anti-LasA 1:2000 (C) was used as a primary antibody; donkey anti-rabbit HRP-coupled as secondary. L = ladder, PAO1 = PAO1 wild-type strain, STM = STM2895, 3401 = STM2895+pMON3401, LasA = lasA::gm, LAsB = ΔlasB::sm, Δ2895 = ΔPA2895::gm, Δ2896 = ΔPA2896::gm.

Fig. 8. Lysine proteases analysis. Poly-L-lysine degradation was measured using 10 µl of overnight culture supernatant incubated in the presence of 40 µg of poly-L-lysine (mol. wt. 500-2000) in 10 mM Tris (pH 8.0), final volume 20 µl. Mixtures were incubated at 50°C for 1 h and then spotted on silica-gel TLC. The plate was chromatographed for 1.5 h in 10:1 ammonium hydroxide - n-propanol. Spots were revealed using ninhydrin spray 0,2% in ethanol. PAO1 - Track 1, STM2895 - Track 2, STM2895+pUCP19 - Track 3, STM2895+pMON3401 - Track 4, , Poly-L-lysine negative control - Track 5.

Table 1. Bacterial strains and plasmids used in this study.

Strains		Genotype or revelant characteristics	Source
E. coli			
	SM10	*thi*-1 *thr leu tonA lacY supE recA* ::RP4-2-Tc ::Mu (Km^R)	de Lorenzo and Timmis, 1994
	DH10B	F^- *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ*80dlacZ*Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara,leu*)*7697 galU galK l-rpsL nupG*	Invitrogen
P. aeruginosa			
	PAO1	Wild type	Holloway, 1955
	STM2895	PAO1; *PA2895*::mini-Tn*5* GFP; Tc^R	Potvin *et al*., 2003
	ΔPA2895::Gm^R	PAO1; Δ*PA2895*::Gm; Gm^R	This study
	ΔPA2896::Gm^R	PAO1; Δ*PA2896*::Gm; Gm^R	This study
	PDO801	PAO1; *lasA*::Gm; Gm^R	Ohman, DE
	PDO240-1	PAO1; Δ*lasB*::Str; Str^R	Ohman, DE
Plasmids			
	pEX18Tc	Tc^R; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18	Hoang *et al*., 1998
	pTZ18R	Ap^R; pUC18, polylinker *lacZ*, *lac*^{iq} selection	
	pUCP19	Ap^R; *Pseudomonas*, *Escherichia coli* shuttle vector	West *et al*., 1994
	pMON3401	Ap^R;1-Kb PA2895 fragment from PAO1 ligated into *Sac*I-*Xba*I site of pUCP19	This study
	pMON3429	Ap^R; 0.7-Kb PA2896 fragment from PAO1 ligated into *Sac*I-*Xba*I site of pUCP19	This study

pMON3438	Ap^R; Gm^R; 1.1-Kb blunt-ended Gm^R *Sma*I fragment of pPS856 ligated between the *AvaI-BsmI* site of pMON3401	This study
pMON3439	Ap^R; Gm^R; 1.1-Kb blunt-ended Gm^R *Sma*I fragment of pPS856 ligated between the *BsmI/ClaI* site of pMON3429	This study
pMON3440	Tc^R; Gm^R; 1.7-Kb blunt-ended Δ*PA2895*::Gm *Hind*III-*Sfi*I fragment of pMON3438 ligated into the *Sma*I site of pEX18Tc	This study
pMON3441	Tc^R; Gm^R; 1.7-Kb blunt-ended Δ*PA2896*::Gm *Hind*III-*Sfi*I fragment of pMON3438 ligated into the *Sma*I site of pEX18Tc	This study

Table 2. Elastolytic activity specific for the LasA enzyme

Strains	OD 595 [a]	ΔOD [b]
PAO1	0.17	0.55
STM2895	0.71	0.01
STM2895 + pUCP19	0.68	0.04
STM2895 + pMON3401	0.13	0.59
PDO801 (lasA::Gm)	0.76	-0.04
PDO240-1 (ΔlasB::Str)	0.40	0.32
ΔPA2895::Gm	0.68	0.04
ΔPA2896::Gm	0.73	-0.01
LB broth [c]	0.72	0

[a] Elastolytic activity was evaluated by a single staphylolytic assay using the cell-free supernatant from a 48 h static culture and a heat-killed *Staphylococcus aureus* suspension. Hydrolytic activity was measured by the decrease of the optical density at 595 nm for 60 min at 37°C.

[b] ΔOD is a comparative value from the negative control LB broth and the supernatant LasA activities. The value given is directly proportional with LasA elastase activity.

[c] The supernatant sample was substituted by LB broth.

Table 3. Elastolytic activity specific for the LasB enzyme

Strains	OD 495 [a]	ΔOD [b]
PAO1	0.66	0.63
STM2895	0.14	0.11
STM2895 + pUCP19	0.14	0.11
STM2895 + pMON3401	0.73	0.70
PDO801 (lasA::Gm)	0.27	0.24
PDO240-1 (ΔlasB::Str)	0.10	0.07
ΔPA2895::Gm	0.15	0.12
ΔPA2896::Gm	0.24	0.21
LB broth [c]	0.03	0

[a] LasB activity was quantitated by a single elastin congo-red assay using the cell-free supernatant from a 48 hours static culture. The degradation of elastin covalently linked to congo-red was measured by increasing of optical density at 495 nm.

[b] ΔOD is a comparative value from the negative control LB broth and the supernatant Las B activity. The value is directly in proportion with LasB elastase activity.

[c] The supernatant sample was substituted by LB broth.

Table 4. Comparative list of genes upregulated in PAO1 ΔPA2896::Gm and STM2895 as determined by one chip hybridization analysis with wild-type strain PAO1.

PA #	Gene	Function/Putative function	Fold change [c]	
			STM2895	**Δ2896::Gm**
PA0173		probable methylesterase		5.3
PA0492		conserved hypothetical protein	4.0	6.1
PA0493		probable biotin-requiring enzyme	9.2	13.9
PA0494		probable acyl-CoA carboxylase subunit	7.0	9.8
PA0495		hypothetical protein	6.5	7.5
PA0496		conserved hypothetical protein	5.7	7
PA0527	*dnr*	transcriptional regulator	8.0	8.6
PA0600		probable two-component sensor	7.0	8
PA1196		probable transcriptional regulator	10.6	13
PA1494		conserved hypothetical protein	55.7	
PA1510		hypothetical protein	7.5	5.7
PA1667		hypothetical protein	6.5	5.7
PA1669		hypothetical protein	6.5	5.3
PA1694	*pscQ*	translocation protein in type III secretion	7.0	5.3
PA1695	*pscP*	translocation protein in type III secretion	13.0	19.7
PA1696	*pscO*	translocation protein in type III secretion	8.0	5.7
PA1699		conserved hypothetical protein in type III secretion	6.1	3.2
PA1701		conserved hypothetical protein in type III secretion	9.2	6.1
PA1706	*pcrV*	type III secretion protein	6.5	4.9
PA1707	*pcrH*	regulatory protein	8.0	5.7
PA1708	*popB*	translocator protein	9.8	6.1
PA1709	*popD*	translocator protein	9.2	5.7
PA1710	*exsC*	exoenzyme S synthesis protein C precursor	9.2	7

PA1711		hypothetical protein	8.6	6.5
PA1712	*exsB*	exoenzyme S synthesis protein B	6.5	3.2
PA1717	*pscD*	type III export protein	9.8	8.6
PA1720	*pscG*	type III export protein	5.3	3.7
PA1901	*phzC2*	phenazine biosynthesis protein	5.3	4.3
PA1902	*phzD2*	phenazine biosynthesis protein	7.0	5.7
PA2110		hypothetical protein	19.7	24.3
PA2111		hypothetical protein	7.0	4.9
PA2113		probable porin	21.1	8
PA2114		probable MFS transporter	9.8	5.3
PA2191	*exoY*	adenylate cyclase	5.7	5.3
PA2193	*hcnA*	hydrogen cyanide synthase	9.2	9.2
PA2194	*hcnB*	hydrogen cyanide synthase	9.8	8
PA2195	*hcnC*	hydrogen cyanide synthase	6.1	5.3
PA2324		hypothetical protein	7.0	
PA2369		hypothetical protein		8.6
PA2593		hypothetical protein		6.1
PA2896		probable sigma-70 factor, ECF subfamily	9.8	
PA2927		hypothetical protein		5.7
PA3187		probable ATP-binding component of ABC transporter	5.7	13.9
PA3188		probable permease of ABC sugar transporter	8.0	14.9
PA3189		probable permease of ABC sugar transporter	14.9	22.6
PA3190		probable binding protein component of ABC sugar transporter	4.9	12.1
PA3327		probable non-ribosomal peptide synthetase	9.8	8.6
PA3328		probable FAD-dependent monooxygenase	8.6	7
PA3329		hypothetical protein	8.6	7.5

PA3330		probable short chain dehydrogenase	9.2	7.5
PA3331		cytochrome P450	7.5	7
PA3332		conserved hypothetical protein	6.5	5.3
PA3333	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	11.3	9.2
PA3334		probable acyl carrier protein	9.2	6.5
PA3335		hypothetical protein	9.2	8
PA3336		probable MFS transporter	9.2	7
PA3724	*lasB*	elastase LasB	8.0	7
PA3841	*exoS*	exoenzyme S	5.7	4.0
PA3842		probable chaperone	6.1	8
PA3843		hypothetical protein	6.5	5.3
PA3905		hypothetical protein	7.0	6.5
PA4211		probable phenazine biosynthesis protein	9.8	8.6
PA4218[b]		probable transporter	6.5	13.9
PA4220[b]		hypothetical protein	8.0	8.6
PA4225[a, b]	*pchF*	pyochelin synthetase	9.2	7
PA4226[b]	*pchE*	dihydroaeruginoic acid synthetase	19.7	13
PA4231[a, b]	*pchA*	salicylate biosynthesis isochorismate synthase	5.7	10.6
PA4348		conserved hypothetical protein	5.7	6.5
PA4495		hypothetical protein	13.0	
PA5033		hypothetical protein	7.0	8.6

[a] [b] PA numbers in bold indicate that these genes were previously demonstrated to be regulated by iron. [a] Genes predicted responding to iron by *Palma M. et al., 2003* [b] Genes involved in response to iron starvation by *Ochsner U.A. et al. 2002*

[c] Fold change in upregulation of mRNA level of STM2895 and PAO1 Δ2896::Gm strains compared to the wild-type PAO1. Values in red indicate that the mRNA level is below 5 fold.

Table 5. Comparative list of genes downregulated in PAO1 ΔPA2896::Gm and STM2895 as determined by one chip hybridization analysis with wild-type strain PAO1.

PA #	Gene	Function/Putative function	Fold change	
			STM2895	**Δ2896::Gm**
PA0160		hypothetical protein	-10.6	-8.6
PA0161		hypothetical protein	-4.9	-10.6
PA0276		hypothetical protein		-5.7
PA0471[b]		probable transmembrane sensor	-7.0	-19.7
PA0472[b]		probable sigma-70 factor, ECF subfamily	-6.5	-11.3
PA0500	*bioB*	biotin synthase	-8.0	-6.5
PA0520	*nirQ*	regulatory protein	-13.0	-14.9
PA0521	*nirO*	probable cytochrome c oxidase subunit	-29.9	-29.9
PA0523	*norC*	nitric-oxide reductase subunit C	-48.5	-39.4
PA0524	*norB*	nitric-oxide reductase subunit B	-27.9	-32
PA0525	*norD*	probable dinitrification protein	-16.0	-16.0
PA0665		conserved hypothetical protein	-5.7	-6.5
PA0672[b]		hypothetical protein	-17.1	-52.0
PA0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase	-4.3	-7.0
PA0905	*rsmA*	regulator of secondary metabolites	-3.2	-5.3
PA0929[b]		two-component response regulator		-5.7
PA1137		probable oxidoreductase	-10.6	-21.1
PA1300[b]		probable sigma-70 factor, ECF subfamily	-8.0	-5.3
PA1301[b]		probable transmembrane sensor	-6.1	-4.3
PA1313		probable MFS transporter	-9.8	-5.7
PA1317	*cyoA*	cytochrome o ubiquinol oxidase subunit II	-4.9	-5.7
PA1319	*cyoC*	cytochrome o ubiquinol oxidase subunit III		-6.1
PA1320	*cyoD*	cytochrome o ubiquinol oxidase subunit IV		-9.2

PA1847		conserved hypothetical protein	-6.1	-6.1
PA2009	*hmgA*	homogentisate 1,2-dioxygenase	-4.0	-6.5
PA2021		hypothetical protein	-3.1	-7.0
PA2033 [b]		hypothetical protein	-4.9	-5.7
PA2384[b]		hypothetical protein		-7.5
PA2398[b]	*fpvA*	ferripyoverdine receptor	-18.4	-22.6
PA2400[b]	*pvdJ*	probable non-ribosomal peptide synthetase	-16.0	-8.6
PA2402[b]		probable non-ribosomal peptide synthetase	-8.0	-3.7
PA2404[b]		hypothetical protein		-6.5
PA2405[b]		hypothetical protein		-11.3
PA2411[b]		probable thioesterase	-8.6	-7.5
PA2412[b]		conserved hypothetical protein	-6.1	-7.5
PA2424[b]		probable non-ribosomal peptide synthetase	-6.1	-13.0
PA2426[b]	*pvdS*	sigma factor	-24.3	-13.9
PA2468[a, b]		probable sigma-70 factor, ECF subfamily	-6.5	-4.3
PA2662		conserved hypothetical protein	-22.6	-24.3
PA2663		hypothetical protein	-36.8	-39.4
PA2664	*fhp*	flavohemoprotein	-512.0	-776.0
PA2686[a]	*pfeR*	two-component response regulator	-11.3	-7.5
PA2691		conserved hypothetical protein	-9.8	-11.3
PA2847		conserved hypothetical protein	-3.2	-5.7
PA3126	*ibpA*	heat-shock protein	-4.9	-7.0
PA3237		hypothetical protein	-7.0	-3.7
PA3430		probable aldolase	-3.7	-5.3
PA3431		conserved hypothetical protein	-6.5	-13.9
PA3432		hypothetical protein	-4.0	-14.9
PA3530[b]		conserved hypothetical protein	-29.9	-39.4
PA3584	*glpD*	glycerol-3-phosphate dehydrogenase	-7.0	-14.9

PA3600		conserved hypothetical protein	-3.7	-5.3
PA3601		conserved hypothetical protein	-4.9	-5.7
PA3811[b]	*hscB*	heat shock protein	-5.7	-4.6
PA3814	*iscS*	L-cysteine desulfurase (pyridoxal phosphate-dependent)	-5.7	-4.6
PA3815		conserved hypothetical protein	-8.0	-4.9
PA3873	*narJ*	respiratory nitrate reductase delta chain	-4.0	-5.3
PA3874	*narH*	respiratory nitrate reductase beta chain	-7.5	-9.2
PA3875	*narG*	respiratory nitrate reductase alpha chain	-13.9	-16
PA3899[b]		probable sigma-70 factor, ECF subfamily	-9.2	-14.9
PA3900[b]		probable transmembrane sensor	-3.5	-8.0
PA3901	*fecA*	Fe(III) dicitrate transport protein	-26.0	-8.6
PA3919		conserved hypothetical protein	-7.0	-8.6
PA3920		probable metal transporting P-type ATPase	-13.0	-18.4
PA3972		probable acyl-CoA dehydrogenase	-5.7	-6.1
PA4090[a]		hypothetical protein	-8.6	-6.1
PA4156[a]		probable TonB-dependent receptor	-6.1	-4.6
PA4158[b]	*fepC*	ferric enterobactin transport protein	-7.0	-21.1
PA4227[b]	*pchR*	transcriptional regulator	-11.3	-9.8
PA4309	*pctA*	chemotactic transducer	-6.1	-5.7
PA4468[a, b]	*sodM*	superoxide dismutase	-13.0	-16
PA4469[a, b]		hypothetical protein	-32.0	-55.7
PA4470[a, b]	*fumC1*	fumarate hydratase	-36.8	-59.7
PA4471[a, b]		hypothetical protein	-137.2	-32
PA4515[b]		conserved hypothetical protein	-5.3	-5.7
PA4542	*clpB*	ClpB protein	-4.0	-5.3
PA4570[b]		hypothetical protein	-26.0	-26.0
PA4615		probable oxidoreductase	-8.0	-8.6

PA4687	*hitA*	ferric iron-binding periplasmic protein	-5.7	-4.6
PA4709[b]		probable hemin degrading factor	-6.1	-9.2
PA4710[a]		probable outer membrane hemin receptor	-3.7	-6.1
PA4783		conserved hypothetical protein	-4.0	-5.3
PA4881		hypothetical protein	-9.2	-12.1
PA4896[b]		probable sigma-70 factor, ECF subfamily	-5.3	-5.3
PA5023		conserved hypothetical protein	-10.6	-11.3
PA5053	*hslV*	heat shock protein	-5.7	-4.9
PA5088		hypothetical protein	-3.5	-14.9
PA5249		hypothetical protein	-6.5	
PA5275		conserved hypothetical protein	-10.6	-10.6
PA5531[b]	*tonB*	TonB protein	-8.0	-7
		Intergenic region between PA0716 and PA0717	-4.0	-11.3
		Intergenic region between PA1372 and PA1373		-21.1
		Intergenic region between PA3860 and PA3861	-3.7	-5.3
		Intergenic region between PA4100 and PA4101	-2.8	-6.1
		tRNA_Alanine		-7.0
		tRNA_Arginine		-7.5
		tRNA_Asparagine		-6.1
		tRNA_Glycine	-4.0	-12.1
		tRNA_Histidine	-4.9	-19.7
		tRNA_Lysine	-6.5	-29.9
		tRNA_Methionine	-4.9	-10.6
		tRNA_Proline		-13.0
		tRNA_Serine		-8.6
		tRNA_Threonine		-5.7
		tRNA_Tyrosine	-3.7	-12.1
		tRNA_Valine	-3.5	-7.5

[a] [b] PA numbers in bold indicate that these genes were previously demonstrated to be regulated by iron. [a] Genes predicted responding to iron by *Palma M. et al., 2003* [b] Genes involved in response to iron starvation by *Ochsner U.A. et al. 2002*

[c] Fold change in downregulation of mRNA level of STM2895 and PAO1 Δ2896::Gm strains compared to the wild-type PAO1. Values in red indicate that the mRNA level is below 5 fold.

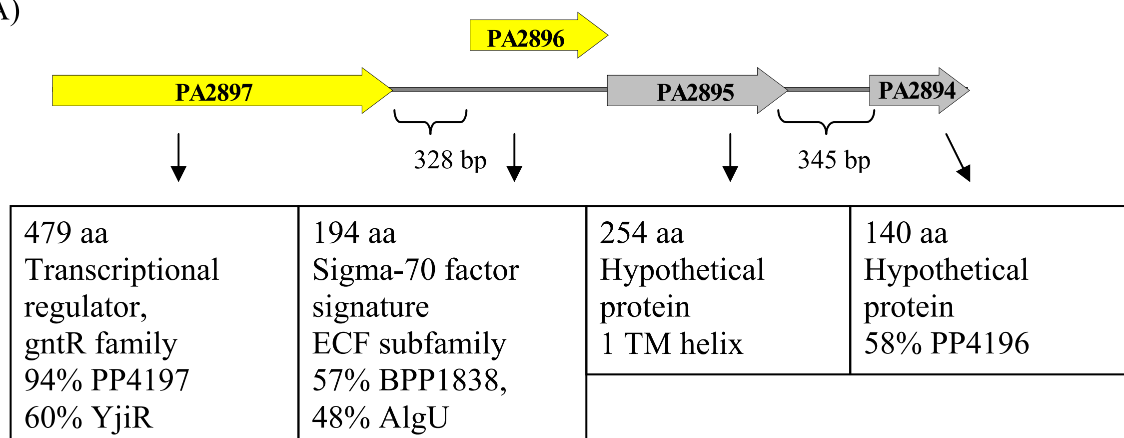
Table 6. Competitive index of the STM2895, PAO1 ΔPA2895::Gm and PAO1 ΔPA2896::Gm strains compare to parent strain PAO1.

Strains	CI [a]
STM2895/PAO1	0.074 ± 0.016
PAO1 ΔPA2895::Gm/PAO1	0.116 ± 0.095
PAO1 ΔPA2896::Gm/PAO1	> 1

[a] Values were calculated in comparison to the wild-type strain PAO1. CI is defined as the output ratio of mutants (CFUs) divided by the output ratio of wild-type PAO1 (CFUs).

Figure 1

A)



B)

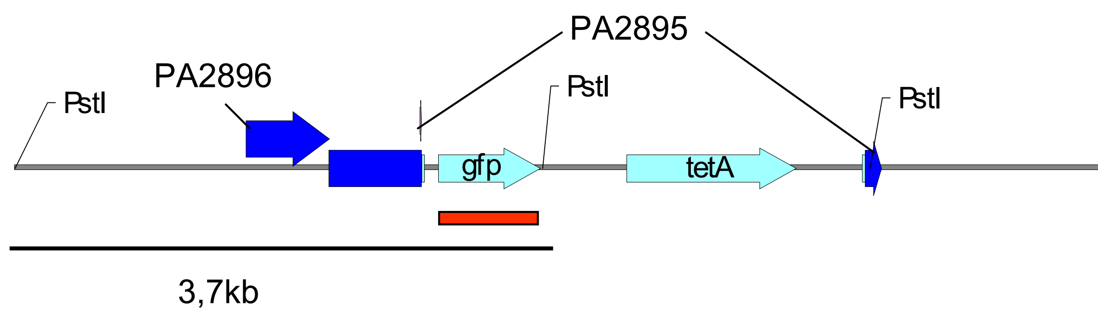
CGCCGGCTGCTGGCCGAGGAGGTGGCGGT**ATGA**ACCCACAGAAGCATTCCGCGAGC

R R L L A E E V A V M* N P Q K H S A S

PA2895

Figure 2

A)



B)

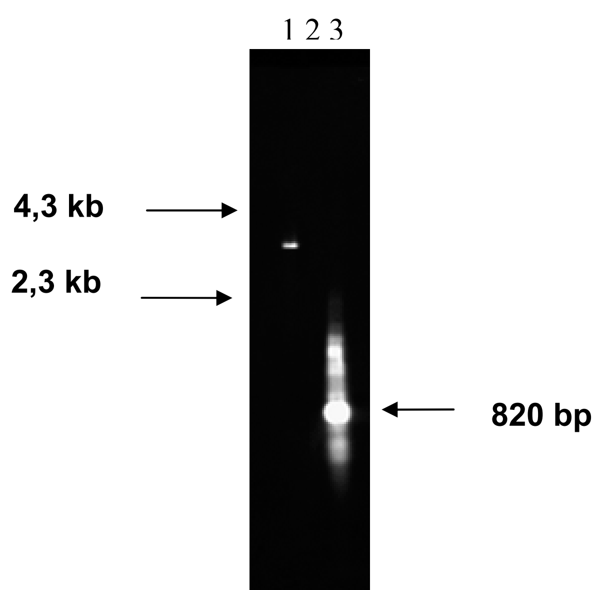


Figure 3

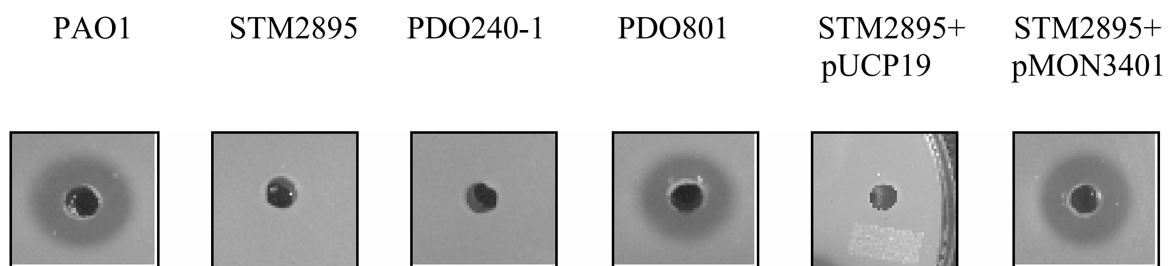


Figure 4

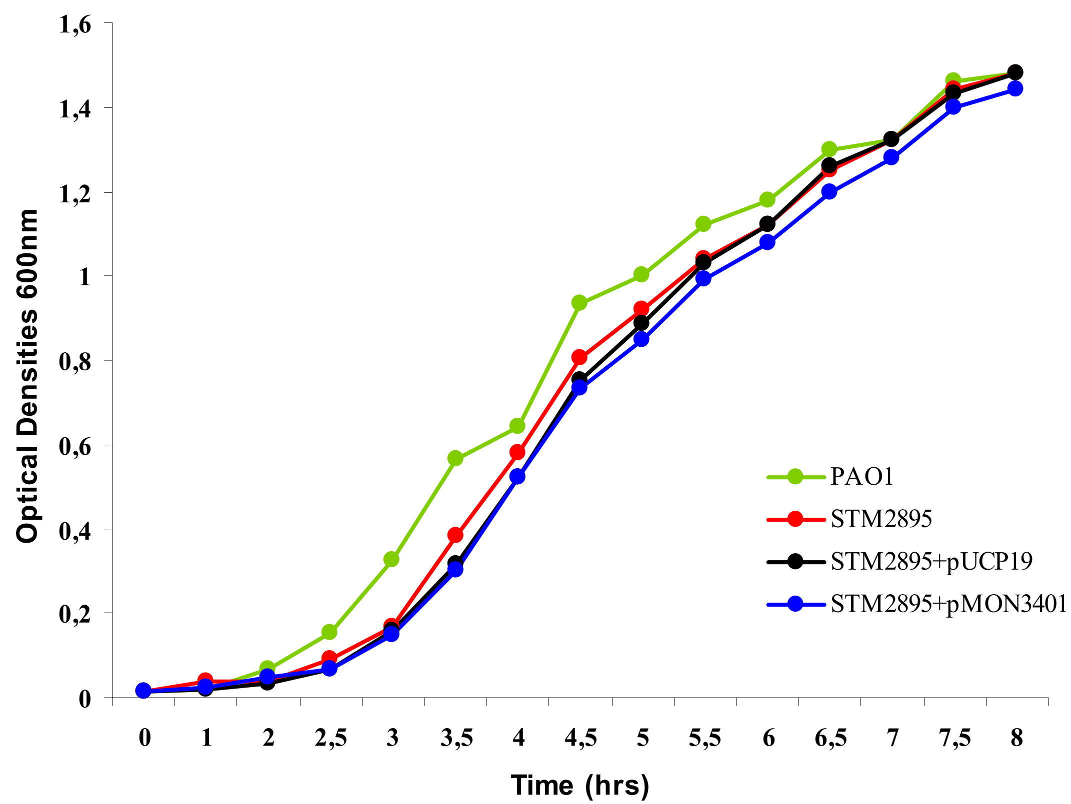


Figure 5

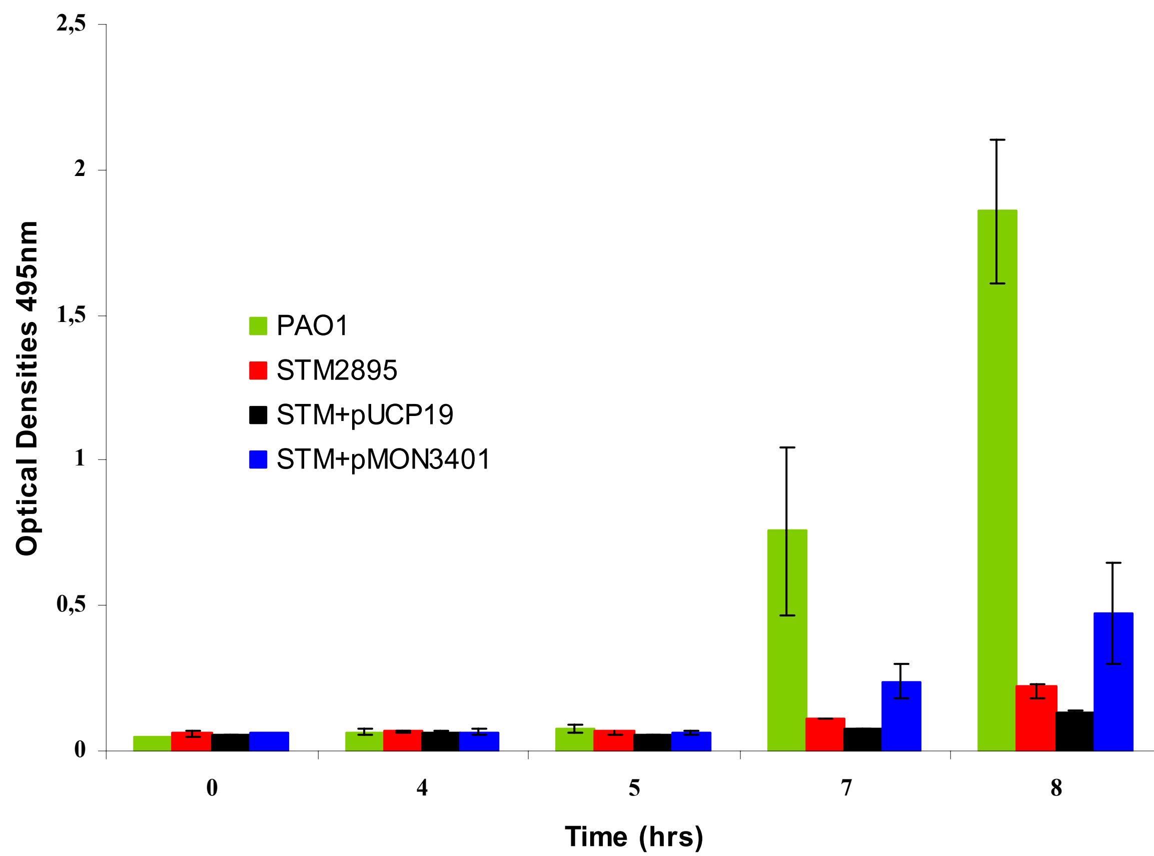


Figure 6

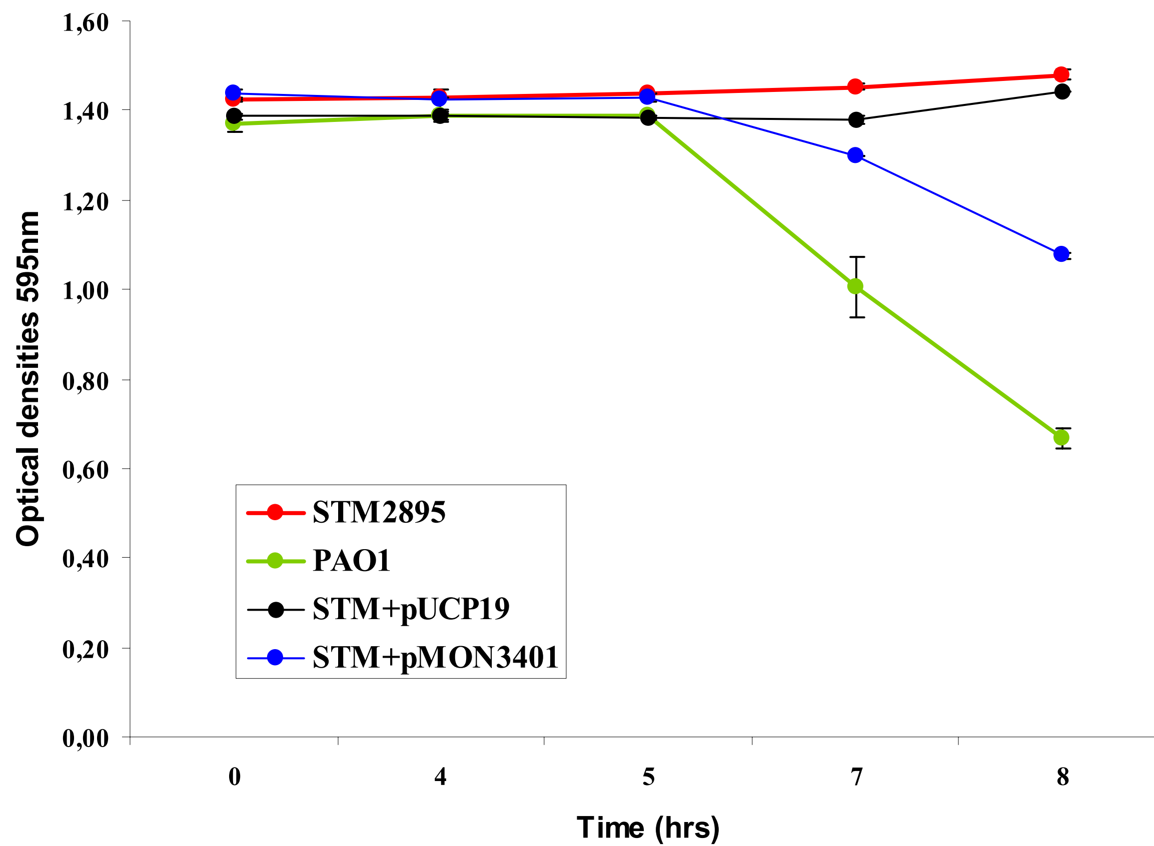


Figure 7

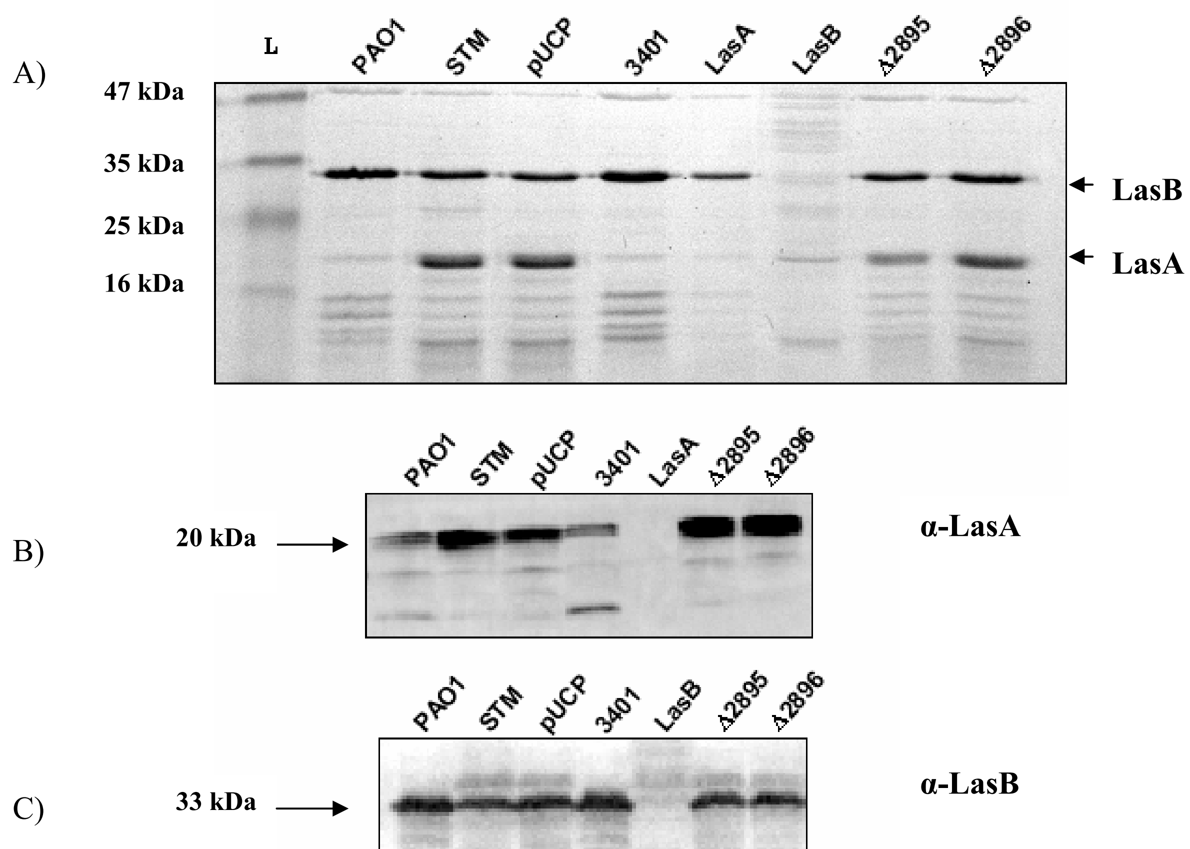
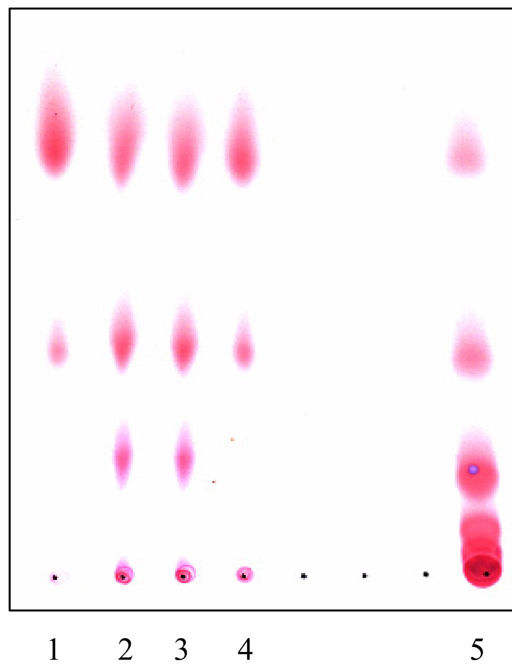


Figure 8



Discussion

La STM chez le rat et le criblage

Chez *Pseudomonas aeruginosa*, un nombre important de facteurs de virulence ont été étudiés à ce jour. Pensons entre autres à toutes les enzymes hydrolytiques produites dans le milieu extracellulaire, les protéines d'attachement, les toxines, l'alginate etc., tous orchestré via le quorum sensing. Cependant, les gènes connus de la virulence ne forment qu'une mince proportion de 1,5 % de tous les cadres de lectures prédits par la séquence de PAO1 (Pseudomonas.com). L'hypothèse évidente que le nombre de gènes impliqués directement dans la virulence de la bactérie soit beaucoup plus élevé que prévu anime le désir d'en approfondir la connaissance. Dans ce projet, nous avons fait appel à la technologie de la STM. La STM est une technologie puissante permettant de cribler plusieurs mutants transpositionnels à la fois dans un seul animal, réduisant ainsi temps et efforts. La STM, utilisant l'hybridation différentielle, a servie maintes fois dans l'identification de facteurs de virulence chez d'autres organismes comme *V. cholerae*, *S. aureus*, *S. pneumoniae*, etc. En utilisant l'amplification par PCR du transposon et de l'étiquette unique, nous avons rendu la technologie beaucoup plus rapide et plus facile à interpréter (Lehoux and Levesque, 1999). De récentes études de recherche de facteurs de virulence à haut débit ont montrées la possibilité d'utiliser le PCR semi-aléatoire permettant d'amplifier directement la jonction transposon-ADN génomique pour un criblage encore plus efficace. De plus, ces nouvelles approches confèrent la possibilité de déterminé le pourcentage de couverture génomique avant le criblage *in vivo* (Bosse *et al.*, 2006). Un des critères cruciaux de la STM est l'utilisation d'un modèle animal fiable et établi. Nous avons décidé d'utiliser le modèle d'infection pulmonaire chronique chez le rat, car il mime bien les conditions du poumon FK. De plus, le modèle des billes d'agar nous procure un modèle d'étude à la fois stable et dynamique d'infection chronique permettant de comparer dans le temps le potentiel infectieux de différentes souches (indice de croissance et indice de compétition).

Plusieurs autres facteurs de la technologie de la STM vont influencer le résultat d'un criblage. La mutagénèse STM repose sur de multiples évènements de transposition et des

scientifiques ont suggérés que les génomes pourraient posséder des sites de prédilection d'insertion du transposon (hot spots). Évidemment, plus il y a de mutants générés dans la banque, plus la couverture génomique sera importante. Par exemple, il aurait fallut générer une banque 10 fois plus importante en nombre de mutants que le nombre de cadres de lecture du génome pour avoir un pourcentage de ciblage de 99% (soit près de 60000 mutants!). Notre criblage ne compte que pour un peu plus d'un génome, soit 7968 mutants versus 5570 ORFs. La représentation graphique des différentes insertions transpositionnelles issues du criblage *in vivo* (voir figure 1, chapitre 3) montre une distribution assez uniforme ormis une importante section d'environ 1 Mb qui ne comporte que 4 insertions rapprochées. Évidemment, cette figure ne tient compte que des gènes essentiels à l'infection testés dans nos conditions expérimentales, il aurait été intéressant de connaître la distribution génomique de toute la banque de mutants. Les produits d'amplification issus d'une combinaison d'amorces, une spécifique au transposon et l'autre aléatoire, permettent d'effectuer des hybridations de type microarray donnant une vue d'ensemble des différents gènes visés par les transposons (Pobigaylo *et al*., 2006). À l'inverse, des groupes de gènes, organisés vraisemblablement en opérons, ont été identifiés comme de nouveaux îlots de pathogénie; par exemple les gènes PA0073, PA0077, PA0082, PA0088, PA0090 et PA0098. La majorité des gènes, entre PA0070 et PA0091 ne possèdent pas de fonction connue, mais seulement des homologies significatives chez d'autres pathogènes comportant un arrangement génique similaire (données non montrées).

Les nouveaux facteurs de virulences

Notre étude s'appuie sur les différents facteurs de virulence connus de *P. aeruginosa* identifiés comme essentiels à l'établissement de l'infection pulmonaire chronique chez le rat. Les gènes codant pour la biosynthèse des pilis (*pilD*, *pilI* et *pilY1*) sont cruciaux pour l'attachement cellulaire, la colonisation et l'infection chez tous les hôtes connus de *Pseudomonas aeruginosa* (Dorr *et al*., 1998; Comolli *et al*., 1999) et via la motilité de type « twitching » (Mattick, 2002). Les gènes responsables de l'hyperproduction de mucus extracellulaire de type alginate sont aussi connus pour leur implication en virulence et

surtout dans l'établissement d'une infection chronique (Ramsey and Wozniak, 2005). Il est cependant surprenant de voir que des produits de gènes impliqués dans la mucoidie semblent essentiels à l'infection étant donné l'enrobage des cellules de *P. aeruginosa* dans l'agarose. Également connu comme facteur de virulence, la protéase alcaline (*aprF*) persiste dans le poumon FK, mais est dispensable dans un modèle de kératite de souris (Suter, 1994; Pillar *et al*., 2000). L'amido-peptidase PepA est nécessaire *in vitro* pour tuer les cellules épitéliales et est également une cause de pneumonie aigue chez la souris. Plusieurs des propriétés de PepA suggèrent qu'elle soit sécrétée via le système de sécrétion de type III (Hauser *et al*., 1998). RhlB est une protéine impliquée dans la synthèse de rhamnolipides (biosurfactants) via le quorum sensing et essentielle aux interactions cellule-cellule est également un facteur de virulence très étudié (Davey *et al*., 2003). Et finalement, *estA* et *wbpX* codent pour une lipase membranaire et une enzyme essentielle à la biosynthèse du LPS (Govan and Deretic, 1996; Daniels *et al*., 2002).

D'autres contrôles internes à notre approche viennent valider la STM : l'identification de mutants auxotrophes présentés ici à cause de leur relation évidente au métabolisme central et intermédiaire (Tableau 1, chapitre 3). Effectivement, il a été démontré que la mutation *purA* (enzyme PurA impliquée dans la biosynthèse des purines) sert de contrôle négatif pour la croissance et le maintien de souches virulentes retrouvées dans le poumon du rat dans la technologie IVET (In vivo expression technology) (Angelichio and Camilli, 2002). Suite aux conjugaisons suicides des transposons, il aurait peut-être été préférable de sélectionner les transconjugants sur milieu minimal. Ceci aurait permis d'éliminer ces gènes d'auxotrophie essentiels *in vivo* mais non impliqué dans la virulence. Une étude chez *V. cholerae* a démontré qu'en co-inoculant un mutant auxotrophe *bioB* ::Tn et la biotine en supplément, on améliorait grandement la croissance dans le modèle de l'infection intestinale aigue chez la souris néonatale (Chiang and Mekalanos, 1998). Les données issues des différentes études d'auxotrophies démontrent les limites nutritionnelles des tissus sains pour les bactéries pathogènes.

Le criblage STM a également permis de faire ressortir de nombreux autres gènes essentiels à l'infection dans le modèle d'infection pulmonaire chronique chez le rat; présentés dans le tableau 2 du chapitre 3. À première vue, plusieurs caractéristiques des différents mutants

atténués sont marquantes. En premier lieu, plus de la moitié des produits des gènes sont localisés au niveau membranaire ou dans l'espace périplasmique. Évidemment, les membranes cellulaires des bactéries à Gram-négatif recèlent d'innombrables protéines d'intérêt en virulence. Par exemple, les protéines des différents systèmes de sécrétion sont incluses dans les membranes internes et externes, des protéines d'interactions avec des récepteurs épithéliaux de l'hôte, des protéines senseurs, des systèmes à deux composantes etc. Bref, les membranes de *P. aeruginosa* sont le haut-lieu d'interactions majeures entre la bactérie et son environnement, conférant ainsi une grande capacité d'adaptation et une grande versatilité nutritionnelle (Stover *et al.*, 2000). Une autre caractéristique importante ressortante de la banque de mutants STM est l'absence d'une bonne cohésion entre les différentes classes de gènes des projets génomes et de celles marquées par le criblage des mutants (voir figure 2, chapitre 3). Par exemple, la classe « facteurs de virulence sécrétés » est surreprésentée, supportant ainsi l'idée que la STM sert à identifier de nouveaux facteurs importants au maintient *in vivo*.

Analyse phénotypique en regard aux facteurs de virulence connus

Les 214 mutants STM incapables de se maintenir dans le poumon du rat ont été analysés de façon systématique pour l'expression de différents facteurs de virulence connus de *P. aeruginosa*. Les tests ont été choisi en fonction de leur rapidité d'exécution et sont dans la quasi-totalité des cas, des tests répertoriés maintes fois dans la littérature. Bien que la liste des tests phénotypiques utilisés dans cette étude soit très exhaustive (8 tests en plus du modèle d'infection chez la drosophile) seulement 36 insertions génomiques (24%) ont été montré ayant un effet sur ces facteurs de virulence. Il est important de mentionner que plusieurs facteurs de virulence n'ont pas été testés (notamment l'hémagglutination, la relation avec le fer, la production de surfactants et le dosage des molécules du quorum-sensing). Le lien ainsi créé entre le phénotype d'un mutant et son incapacité à se maintenir *in vivo* en fait un mutant de choix pour des caractérisations futures. Une des caractéristiques intéressantes de cette liste (tableau 2, chapitre 3) est la redondance des phénotypes liés à la

motilité. Effectivement, 20 mutants sur 36 sont affectés pour la motilité de type « swarming », 4 de type « twitching » et 2 de type « swimming ». La capacité à se déplacer de *Pseudomonas aeruginosa* lui confère des avantages importants comme l'aquisition plus efficace de nutriments, l'éloignement des substances toxiques, la dispersion dans l'environnement et la colonisation d'un hôte (Rashid and Kornberg, 2000). Appliquée à notre modèle d'infection pulmonaire chez le rat dans un groupe de 72 mutants différents, la capacité à se mouvoir devient cruciale, surtout parce que les cellules sont incluses dans des billes d'agarose. Effectivement, les souches incapables de motilité sont reconnues comme étant déficientes en virulence (Mattick *et al*., 1996; Sonnleitner *et al*., 2003). De plus, à l'inverse des facteurs de virulence sécrétés à l'intérieur du poumon, la complémentation en trans de la motilité bactérienne n'est pas possible. D'autres phénotypes intéressants ont été dénotés, tels que l'incapacité de la souche STM2895 à produire des exoprotéases (souche faisant l'objet d'une caractérisation moléculaire plus fine au chapitre 4) et la souche STM0151 étant incapable d'initier la formation d'un biofilm adéquat.

P. aeruginosa STM2895 : un mutant de choix, déficient en virulence

Le criblage de la banque STM nous a fourni plusieurs pistes permettant de poursuivre la caractérisation des mutants et ainsi confirmer l'atténuation de la virulence *in vivo.* Le chapitre 4 présente plusieurs expériences, dont les résultats ont mené à la caractérisation préliminaire du mutant *P. aeruginosa* STM2895 et à la proposition d'un modèle intégré de la régulation génomique. Les critères permettant d'arrêter un choix sur *P. aeruginosa* STM2895 sont sans contredits justifiables. En effet, la présence de 11 isolats STM2895 sur 214 (regroupés sur deux évènements transpositionels différents) renforçait l'hypothèse de l'atténuation *in vivo* de la souche ayant une insertion dans le gène PA2895 avant même la confirmation de l'atténuation de la virulence par l'indice de compétition. De plus, l'absence d'activité protéolytique dans le surnageant de culture de STM2895 suggérait plusieurs indices quant aux causes de cette atténuation. L'essai utilisé pour mettre en évidence le phénotype des protéases est une mesure semi-quantitative qui repose sur la dégradation de

la β-caséine contenue dans le lait (voir matériel et méthodes, chapitre 4). La quasi-totalité des surnageants de culture des mutants testés ont démontré une zone d'hydrolyse égale ou légèrement inférieure à la souche sauvage PAO1. L'expérience ayant été réalisée selon les conditions de culture mises au point par Laux et collaborateurs (2002) c'est-à-dire 48 heures de culture en absence d'oxygénation, contournant les retards de croissance et permattant une production enzymatique maximale. L'absence totale d'une zone d'hydrolyse sur gélose au lait par les enzymes de la souche STM2895 a rapidement gagné notre intérêt pour la suite du projet. Effectivement, la dégradation de la caséine du lait est surtout due à l'action de la protéase LasB ou l'élastase B (Morihara and Homma, 1985). LasB est connue comme étant l'exoprotéase la plus abondante du surnageant chez *P. aeruginosa* (McIver *et al.*, 2004) et considérée comme un de ses principaux facteurs de virulence (Woods *et al.*, 1982).

L'activité élastolytique de STM2895

Nous avons d'abord confirmé le résultat des géloses au lait par une technique quantitative à l'élastine Rouge-Congo (Rust *et al.*, 1994) spécifique à l'activité de l'élastase B. L'élastine Rouge-Congo repose sur une méthode colorimétrique où le substrat d'élastine (des ligaments bovins) est lié de façon covalente au colorant. Les données d'activité élastolytique obtenues avec les surnageants des cultures de 48 hrs sont présentées au tableau 3 du chapitre 4. Les résultats sont en accords avec ceux des géloses au lait, indiquant que le mutant STM2895 est déficient dans la production extra-cytoplasmique d'élastase B. Par contre, la faible reproductibilité statistique de l'essai d'élastine Rouge-Congo sur les cultures de 48 hrs, nous a poussé à démontrer le phénotype via l'induction de l'enzyme en courbe de croissance, où l'inoculum de départ et la densité bactérienne sont contrôlés. Les courbes de croissance réalisées pour le mutant STM2895 et pour une souche complémentée *en trans* par l'ORF de PA2895 démontre bien le chevauchement des courbes, n'indiquant aucun défaut de croissance, qui aurait pu être à l'origine du phénotype LasB (figure 4, chapitre 4). Tel que démontré auparavant par Brint et Ohman (1995), c'est à la fin de la phase logarithmique et au début de la phase stationnaire de croissance que

l'élastase B est induite par le quorum-sensing (entre 0,8 et 1,0 de densité optique à 600nm) (figure 5, chapitre 4). On peut voir également que la complémentation *en trans* de l'activité LasB via plasmide recombinant pMON3401 (pUCP19 + PA2895) est partielle pour les analyses faites sur une courte période de temps de culture, tandis que la complémentation était totale avec les surnageants de 48 hrs. Ces observations semblent indiquer un problème au niveau de la régulation de la transcription pour le gène PA2895 exprimé *en trans*. Donc, les conditions de culture et la densité optique ont beaucoup d'influence sur l'activité élastolytique.

L'activité élastolytique de *P. aeruginosa* est principalement liée à l'expression de *lasB*, mais une deuxième élastase (LasA) est également produite conjointement via le quorum-sensing (Toder *et al*., 1991). LasA fut démontrée importante pour l'activité élastolytique globale en générant les premières digestions et en permettant un meilleur accès à l'élastine (Peters and Galloway, 1990). Par contre, il est impossible de bien mesurer l'activité de LasA sur un substrat d'élastine. Nous avons donc utilisé un test quantitatif bien décrit dans la littérature reposant sur la dégradation du pont pentapeptidique du peptydoglycan de cellules de *Staphylococcus aureus* inactivées à la chaleur : l'essai de staphylolyse (Kessler *et al*., 1993). C'est la diminution de la densité optique à 595nm de la suspension de cellules de staphylocoque tuées à la chaleur qui est mesurée, exprimant ainsi l'activité spécifique LasA. Les résultats obtenus en utilisant des surnageants de culture de 48 hrs pour l'analyse de la staphylolyse (présentés au tableau 2, chapitre 4) démontrent l'absence totale d'activité LasA par la souche STM2895. Les données obtenues pour la culture de la souche sauvage PAO1 en courbe de croissance montrent aussi l'induction de l'enzyme en fin de phase logarithmique (figure 6, chapitre 4). Ces courbes montrent aussi l'absence totale d'une activité de l'exoprotéase LasA dans le surnageant de STM2895 et d'une complémentation partielle du plasmide en trans pMON3401 comme c'est le cas avec l'élastine Congo-red. En résumé, l'activité des deux élastases LasA et LasB est nulle dans le surnageant de culture de *P. aeruginosa* STM2895 expliquant en partie le déficit de maintient *in vivo* de cette souche. D'autres protéases sont présentes dans le surnageant de culture de PAO1 ayant d'autres cibles que l'élastine, la sérine-protéase IV et la métalloprotéase alcaline. Ces deux exoprotéases ont été démontrées responsables de la dégradation de plusieurs molécules du complément, de cytokines pro-inflammatoires et de d'autres protéines du

tissu conjonctif comme le fibrinogène et le plasminogène (Hong and Ghebrehiwet, 1992; Engel *et al*., 1998). L'article publié par Caballero et collaborateurs (2001) fait état d'une méthode qualitative intéressante sur la mesure spécifique de l'activité de ces deux protéases dans les surnageants de culture : la dégradation de la poly-L-lysine. Le polypeptide composé essentiellement de lysine est incubé en présence du surnageant et ensuite séparé par chromatographie sur gel de silice (voir matériel et méthodes chapitre 4). La disparition des taches (spots) rapprochées du point de dépôt sur le chromatogramme correspondant aux polymères de haut poids moléculaire témoigne d'une activité hydrolytique. L'analyse du chromatogramme de dégradation de la poly-L-lysine réalisé avec le surnageant de 48 hrs de la souche STM2895 versus celui de PAO1 indique effectivement une activité diminuée pour STM2895 (figure 8, chapitre 4). L'absence de souches mutées contrôles pour la protéase IV et la protéase alcaline ne permettent pas d'attribuer cette faible activité à une enzyme spécifique. L'article de Caballero prévoit que l'ajout d'inhibiteurs spécifiques dans la réaction (le TLCK pour la protéase IV et l'EDTA pour la protéase alcaline) permet de vérifier l'activité spécifique à chacune de celle-ci. Cependant, dans le cas des surnageants de culture de STM2895 et de PAO1, l'utilisation de ces inhibiteurs n'a pas permis de conclure à l'implication spécifique d'une de ces enzymes. Ceci suggère donc que d'autres protéases (en plus des élastases A et B) sont touchées par la mutation de PA2895.

Lorsque nous avons comparé l'effet de la mutation de PA2895 sur la sécrétion d'exoprotéases avec une souche mutée dans les gènes *lasB* et/ou *lasA*, l'hypothèse de l'absence des enzymes dans le surnageant de STM2895 était la plus plausible. Effectivement, les étapes de clivage et de repliement de ces deux enzymes sont nombreuses et bien connues. D'abord synthétisées en pré-pro-enzyme, le polypeptide subira deux clivages successifs permettant la translocation de l'enzyme mature vers l'extérieur de la cellule liée à un propeptide stabilisant la protéase mais sans activité dans l'espace périplasmique (McIver *et al*., 2004). Les élastases LasA et LasB utilisent le système de sécrétion de type II (SSTII) ou la voie de sécrétion générale. Le polypeptide nouvellement synthétisé sera pris en charge par les enzymes Sec via le prédomaine de la protéine (leader peptide) et ensuite via Xcp pour la translocation périplasmique et les étapes finales de repliement (Akrim *et al*., 1993). De plus, les enzymes DsbA et DsbC (disulfide bond isomerase) présentes dans l'espace périplasmique sont essentielles à la sécrétion

d'élastases, de lipases et de phospholipases actives et de toutes les enzymes extracellulaires passant par le SSTII (Urban *et al*., 2001). La meilleure façon de vérifier le fonctionnement du SSTII dans la souche STM2895, était de montrer la présence ou l'absence des enzymes LasA et LasB dans le surnageant par immunodétection sur gel dénatrurant. La figure 7 du chapitre 4 nous montre les résultats des immunobuvardages sur les quelques souches clés. La présence de protéases LasA et LasB sous leur forme fonctionnelle (20 kDa pour LasA et 33 kDa pour LasB) dans le surnageant de STM2895 a permis d'écarter toutes les hypothèses liées aux problèmes de sécrétion et de clivage. Nous favorisons donc les hypothèses de mauvais repliement et d'absence des cofacteurs. L'activité optimale et la préparation efficace des deux élastases dépend de la présence d'ions divalents dans le milieu de culture (Olson and Ohman, 1992). Malheureusement, l'ajout de calcium, de fer, de magnésium, de zinc ou de manganèse dans le milieu de culture de STM2895 n'a pas influencé l'activité élastolytique contenu dans les surnageants. La seule hypothèse était celle d'un problème de potentiel d'oxydo-réduction (redox) du périplasme ne permettant pas le repliement correct des enzymes à action élastolytique. L'ajout de DTT ou de peroxyde d'hydrogène pendant l'incubation et après dans le but de faire varier le potentiel redox des cellules STM2895, est demeuré sans effet notable sur l'activité élastolytique. Par contre, la surproduction *en trans* d'une oxydoréductase putative, PA3498, à l'intérieur de la souche STM2895 a permis de rétablir partiellement le phénotype LasA et LasB. Ces données font l'objet d'analyses complètes dans le mémoire de maîtrise de Mme Karine Richard, étudiante du Dr Roger C Levesque.

La fonction probable d'anti-sigma ECF

Des analyses approfondies en bioinformatique n'ont pas permis de trouver une protéine homologue significative connue dans tous les différents projets génomes bactériens en cours ni aucun motif protéique conservé. Ces résultats supportent l'hypothèse que PA2895 serait un gène unique à *P. aeruginosa* et qu'aucun rôle biologique connu ne lui serait attribué, si ce n'est d'être ancré dans une membrane. La poursuite de la caractérisation du mutant STM2895, incapable de se maintenir *in vivo* et incapable de produire des

exoprotéases, s'est dessinée autour de sa cotranscription évidente avec un gène conservé comportant un motif facteur sigma-70 de type ECF (extracytoplasmique function) et une identité de 31% avec FecI chez *E.coli* le régulateur du métabolisme du fer. Plusieurs tentatives infructueuses de démontrer la co-transcription de PA2895-PA2896 par analyses Northern chez la souche sauvage PAO1 nous ont poussé à conclure que le transcrit est très faiblement exprimé dans les conditions normales de culture. Les expérimentations réalisées dans le passé sur les facteurs sigma de type ECF (revue par Raivio and Silhavy, 2001) a permis de suggérer que PA2895 agirait comme facteur anti-sigma sur PA2896. Premièrement, les facteurs sigma sont co-transcrits avec leur facteur anti-sigma ancré dans la membrane cytoplasmique. L'organisation génomique et les analyses bioinformatiques de PA2895-PA2896 supportent ces caractéristiques (voir figure 1, chapitre 4). Deuxièmement, ce sont des sous-unités alternatives de la RNA polymérase impliquée dans la réponse à un stress extracellulaire (ECF, ExtraCellular Function). Dans le cas de PA2895-PA2896, le repliement d'exoprotéases qui dépendent du potentiel redox périplasmique. La découverte de plusieurs stress périplasmiques (température, salinité, pH, radicaux libres, etc.) étant à l'origine de la régulation alternative dépendante de facteurs sigma ECF inclut entre autres le défaut de repliement de protéines périplasmiques à action extracellulaire (Erickson and Gross, 1989; Raivio and Silhavy, 2001). L'espace périplasmique est connue pour être une région à fort potentiel oxidatif, contrairement au cytoplasme qui est réducteur et favorise la génération des ponts di-sulfures (Oliver, 1996). L'espace périplasmique des bactéries à Gram-négatif est exposé constamment aux variations environnementales étant donné la bonne perméabilité de la membrane externe. Les systèmes de types ECF existent pour transloquer un signal vers le cytoplasme pour amener la cellule à répondre aux changements environnementaux (Oliver, 1996). Dans le but d'élucider l'implication de PA2895 et PA2896 dans le contrôle du potentiel redox, nous avons emprunté la voie de la caractérisation du régulon transcriptionnel de l'opéron par l'approche des micropuces à ADN.

L'analyse transcriptionnelle du régulon PA2895-PA2896

La micropuce *P. aeruginosa* souche PAO1 d'Affymetrix contient 5900 oligonucléotides (spots) au total, représentant 5769 sondes de PAO1, 5570 cadres de lecture ouverts annotés, les gènes codant pour les ARNr et ARNt et 199 régions intergéniques. De plus, 117 sondes représentent des gènes ne provenant pas de PAO1, des gènes de transposons, des gènes de résistances aux antibiotiques, alors que 14 sondes servent d'ADN contrôles provenant de *Bacillus subtilis*, *Saccharomyces cerevisiae* et *Arabidopsis thaliana*. Nous avons utilisé la micropuce d'Affymetrix pour étudier les changements dans l'expression globale des mutants STM2895 et PAO1 ΔPA2896 ::gm versus la souche sauvage PAO1. Les résultats présentés aux tableaux 4 et 5 (chapitre 4) proviennent d'une seule hybridation pour chaque souche comparées entre elles. Évidemment, il s'agit de données préliminaires, ces expériences devront être refaites pour en vérifier la reproductibilité. Pour les souches STM2895 et PAO1 ΔPA2896::gm, nous avons noté 128 gènes (64 plus et 64 moins) et 138 gènes (59 plus et 79 moins), 4 régions intergéniques et 12 régions codantes pour des ARNt lesquelles étaient différentiellement régulée par $\geq$ 5 fois (la description complète des différents changements apparaît au chapitre 4). Lorsque comparées entre elles, les données provenant des deux mutations sont très similaires. Effectivement, la cassette de résistance insérée dans le cadre de lecture partiellement délété de PA2896 provoque forcément un effet polaire sur PA2895, générant un mutant double. Donc, l'effet de l'inactivation simple de PA2895 ou des deux gènes cause sensiblement le même effet, comme c'est souvent le cas, tel que le suggère la revue sur les facteurs anti-sigma de Hughes et mathee (1998). Les données obtenues des résultats des transcriptomes ont permis de construire un modèle de régulation associant le phénotype d'exoprotéases et le métabolisme oxydatif du fer. Effectivement, les gènes aux tableaux 4 et 5 notés en caractères gras ont déjà été identifiés à la déficience en fer (Ochsner. *et al*. 2002; Palma. *et al*., 2003). Il a été démontré que l'acquisition du fer est négativement contrôlée via le répresseur Fur qui bloque la transcription des gènes cibles lorsque la concentration d'ions Fe 2+ est suffisante (Neilands, 1990). Alors que le facteur sigma alternatif de type ECF, PvdS, est nécessaire à la transcription des gènes de biosynthèse de la pyoverdine (Vasil and Ochsner, 1999). Il

semble y avoir une forte synergie d'expression entre les différents facteurs sigma ECF de *P. aeruginosa* car le fait de déléter PA2895-PA2896, apporte un effet négatif sur plusieurs autres opérons du même type (voir tableau 5, chapitre 4), engendrant des conséquences importantes sur PvdS par exemple. Les profils transcriptionnels des gènes liés au métabolisme du fer sont très semblables à ceux observés par Ochsner et collaborateurs (2002) où la biodisponibilité du fer a été réduite d'une part et *pvdS* muté d'autre part. Donc, les résultats des transcriptomes sur PA2895-PA2896 tendent à reproduire ceux d'une réponse en absence de fer, sensiblement causée par l'absence de PvdS. La souche STM2895 dénote également une forte induction des gènes du système de sécrétion de type III (SSTIII) contrôlant les exoenzymes Y et S ainsi que plusieurs gènes de virulence comme pour la biosynthèse des phénazine et des cyanures d'hydrogènes (composés toxiques pour les cellules eucaryotes). Plusieurs observations tendent à indiquer que le mutant STM2895 possède un métabolisme de l'oxygène altéré. Effectivement, la cyanogénèse (gènes *hcn* et *phz*) chez *P. aeruginosa* et *Pseudomonas fluorescens* se produit lorsque la concentration en oxygène diminue (Castric, 1983). De plus, le processus de production de ces composés dépend de la conversion vers une forme active du régulateur transcriptionnel ANR (anaerobic regulator of arginine deiminase and nitrate reductase) en absence d'oxygène (Castric, 1994). Bien que ANR ne soit pas présent dans la liste des gènes altérés par la mutation PA2895-PA2896, un régulateur très apparenté à ANR, DNR (dissimilatory nitrate respiration regulator) appartenant à la superfamille CRP-FNR (cAMP receptor protein-fumarate and nitrate reductase regulator) est induit par la présence des nitrites (Rinaldo *et al.*, 2005). Les nitrites font partie des composés utilisés par *P. aeruginosa* comme accepteur final d'électrons. En absence des enzymes de dénitrification, Nar, Nir et Nor, le NO s'accumule dans la cellule (Arai *et al.*, 1999). Des études ont montré que la détoxification du NO en conditions anaérobiques est assurée par les flavohémoglobines (Arai *et al.*, 1995). Donc, il est logique de penser que cette situation s'applique au mutant STM2895 étant donné l'importante répression (-600 fois en moyenne) d'une flavohémoprotéine (PA2664) et des opérons *nar*, *nir* et *nor* (tableau 5, chapitre 4). En conclusion, les données soutiennent un modèle de régulation, certes imparfait, mais logique où la délétion d'un signal périplasmique venant de PA2895 inhibe la réponse de PvdS au manque de disponibilité du fer. Aisni, il y a activation du SSTIII, de facteurs de virulence

(inactifs?) et du ferri-sidérophore alternatif (la pyocheline) dans le but de surpasser ce problème. A l'intérieur de la cellule, la détoxification du NO et des radicaux libres issu de la respiration oxygénique et anoxygénique est hautement réprimée, créant un environnement périplasmique inopportun au repliement correct des exoprotéases.

Conclusion

Les chapitres qui ont été présentés dans cette thèse décrivent l'idée directrice ainsi que l'approche systématique qui a permis d'attribuer une fonction probable à un gène préalablement inconnu. *Pseudomonas aeruginosa* est un pathogène opportuniste causant principalement des infections pulmonaires chroniques chez les patients atteints de la Fibrose Kystique ainsi que différentes infections nosocomiales. Les déterminants génétiques permettant l'établissement d'une infection opportuniste sont peu connus, ainsi l'achèvement du séquençage du génome de *P. aeruginosa* ainsi que la disponibilité de plusieurs modèles animaux d'infection en font un excellent modèle d'étude. Utilisant la technique de mutagénèse à étiquette (STM), nous avons construit une imposante banque de 7968 mutants transpositionels chez *P. aeruginosa*. La STM est une technique basée sur une sélection négative de mutants après passage dans un modèle animal. La STM permet ainsi l'identification de nouveaux facteurs de virulence ainsi que de nouveaux gènes essentiels *in vivo*. Tous les mutants inclus dans cette banque ont été divisés en 96 groupes de 72 mutants dont chacun de ces mutants possède une identité moléculaire qui lui est propre (3 transposons x 24 étiquettes). Le criblage de ces 96 groupes de mutants, dans le modèle d'infection pulmonaire chronique utilisant les billes d'agar, a permis de ressortir 243 mutants incapable de maintient *in vivo*. De ces 243 mutants, l'identité de 214 a été révélé suite au séquençage de la jonction ADN génomique/mini-transposon à la suite du clonage d'un fragment *PstI* et d'une sélection dépendante du marqueur de résistance du mini-transposon. Les banques de données du projet génome de *P. aeruginosa* ont permis de restreindre ces 214 mutants à 148 gènes différents tous essentiels *in vivo*.

Des analyses phénotypiques ont permis de mettre en évidence de nouveaux gènes affectant la production de facteurs de virulences connus de la bactérie. Un de ceux-ci STM2895, une souche incapable de produire des protéases extracellulaires. L'analyse du contexte

génomique de PA2895 a révélé la présence, sur le même transcrit, du gène PA2896 codant pour un facteur sigma-70 probable de la sous-famille des facteurs ECF (extracytoplasmic functions). Des analyses bioinformatiques sur le gène PA2895 n'ont révélés aucune fonction prédite ainsi qu'aucune protéine possédant une homologie significative chez d'autres espèces. Des analyses de domaines ont cependant prédit une hélice trans-membranaire. Des recherches littéraires concernant les facteurs sigma ECF ont permis d'associer l'opéron PA2895-PA2896 à deux caractéristiques communes des facteurs ECF comme l'inclusion membranaire probable de PA2895 qui pourrait agir comme facteur anti-sigma ainsi que le contrôle de fonctions extracellulaires (protéases). À l'aide de tests biochimiques spécifiques à deux des quatres protéases extracellulaires de *P. aeruginosa*, les élastases LasA et LasB, nous avons démontré qu'au moins ces deux enzymes sont inactives dans le surnageant de culture de la souche STM2895 ainsi que les souches de délétion PAO1 ΔPA2895 ::Gm et PAO1 ΔPA2896 ::Gm. Dans le but de vérifier la présence, le bon traitement et repliement de LasA et LasB dans le surnageant de la souche STM2895, des immunobuvardages de type western ont été réalisés avec des anticorps spécifiques aux deux enzymes. Les analyses en western couplées aux données d'activités des enzymes ont révélé que LasA et LasB sont présentes et bien clivées dans le surnageant de culture mais dans une forme inactive.

Pour terminer, nous avons émis l'hypothèse que le mauvais repliement des protéases extracellulaires était dû à l'absence d'un signal périplasmique dépendant de PA2895 dérégulant ainsi la transcription dépendante de PA2896. Pour vérifier cette hypotyhèse nous avons comparé les profils transcriptionels des souches STM2895 et PAO1 ΔPA2896 ::Gm à celui de la souche sauvage PAO1. À notre grande surprise, les patrons de régulation transcriptionnels étaient très comparables entre les deux mutants. Nous avons donc conclu que l'effet sur la transcription globale était surtout dû à l'absence de PA2895 plutôt qu'une transcription non-contrôlée de PA2896. Les données révélées par les études en transcriptome ont montré que plusieurs gènes préalablement associés au métabolisme du fer sont réprimés dans les souches STM2895 et PAO1 ΔPA2896 ::Gm. De plus, la répression des protéines essentielles au transport de l'oxygène et d'autres impliquées dans la détoxification de radicaux libres suggère que le mauvais repliement des élastases serait dû à une variation du potentiel redox périplasmique. Le périplasme est connu pour être le

lieu où les ponts disulfures nécessaires au bon repliement des enzymes extracellulaires s'effectue, et ce de façon dépendante du potentiel redox. L'absence d'une réponse adéquate à la privation du fer combinée au mauvais repliement des élastases LasA et LasB sont certes la cause de l'atténuation *in vivo* du mutant STM2895 tel que confirmé par les indices de compétition.

Il y a incontestablement un immense travail qui a été fait dans la caractérisation des gènes PA2895 et PA2896 et de leurs implications dans le processus du maintien *in vivo* de *P. aeruginosa*. Par contre, beaucoup d'hypothèses subsistent encore et doivent être confirmées. Entre autres, l'interaction protéine-protéine des deux composantes régulatrices sigma-anti-sigma devra être démontrée via la technique du double-hybride chez la levure ou du tap-tag (Puig *et al*., 2001), l'inclusion de PA2895 dans la membrane cytoplasmique via des fusions transcriptionnelles visant la création de protéines chimériques LacZ ou PhoA, la caractérisation exacte du signal responsable de l'activation transcriptionnelle dépendante de PA2896 ainsi que l'effet de la suppression de PA2895 sur les autres enzymes extracellulaires de *P. aeruginosa* sont toutes des questions auxquelles les réponses ne sont pas encore claires. De plus, le rôle exact de PA2896 sur le métabolisme du fer et comment la balance de ces facteurs sigma ECF à l'intérieur de la cellule se maintient. Est-ce que PA2895 est capable d'intéragir avec PvdS, et comment cette interaction influence la réponse au fer. Évidemment, plusieurs des caractéristiques moléculaires de PA2895 en font une cible thérapeutique de choix. En effet, PA2895 semble être une protéine membranaire unique à Pseudomonas aeruginosa. Il serait intéressant de cribler la protéine purifiée PA2895 dans une banque de petites molécules pour mettre en évidence des interactions avec de potentielles nouvelles drogues ou via une banque peptidique utilisant le phage display.

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Appendice 1

Identification of Novel Pathogenicity Genes by Signature-tagged Mutagenesis and Related Technologies

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Introduction

Microbial pathogens possess a repertoire of virulence determinants that make unique contributions to bacterial fitness during an infection. Understanding the expression of these genes involved in infection remains the holy grail of research in bacterial virulence. The simultaneous development of molecular biological technologies including recent progress in microbial genomics and bioinformatics coupled to molecular genetic bacterial approaches has facilitated the dissection of a plethora of regulatory systems implicated in virulence (1). However, the complexities of bacterial gene expression *in vivo* during mammalian infection cannot be addressed solely by *in vitro* experiments coupled to *in vivo* analysis of a single gene. The infected host presumably represents a complex and dynamic environment, which is modified in a time and dose-dependent manner during the infection process. One may assume then that the bacterial pathogen must turn on or off genes specific for the conditions encountered *in vivo* via a variety of stimuli. In addition, several discrete cellular steps presumably permit the controlled expression of genes essential for initiation of the infection process and its maintenance. The pattern of bacterial genes expressed solely *in vivo* and genes essential for infection remains to be investigated if one wants to have a global vision of the infection versus the pathogen genomic content. This will be a formidable task requiring several complementary techniques such as *in vivo* expression technology (IVET), signature-tagged mutagenesis (STM), elegant bioinformatics analysis and functional genomics analysis via proteomics using 2D gel electrophoresis differential display, DNA and protein arrays and chip technology (1). There is also an urgent need for new technologies for analysis of genes expressed *in vivo* as the current tools have significant limitations. In this chapter, we focus on the recent progress and adaptations of signature-tagged mutagenesis (STM).

STM can now be used in any typical laboratory; the screening can now be done rapidly by automated high throughput robotics using polymerase chain reaction (PCR) instead of hybridization. The design of tags has been simplified and several different miniTn*5*s with a unique phenotypic selection for each can now be utilized. These modifications of STM developed in our laboratory have been applied to *Pseudomonas aeruginosa* PAO1 whose 6.3 Mb genome has been completely sequenced (2). The PCR-based STM is described in

detail including the strategies and protocols using as prototype the *P. aeruginosa* STM project on-going in our laboratory.

Description of signature-tagged mutagenesis

STM is an elegant mutation-based screening method using a population of bacterial mutants for the simultaneous identification of virulence genes of microbial pathogens by negative selection (3). The technique depends upon an *in vivo* selection phenotype whereas genes inactivated will have an effect on diminishing bacterial virulence tested in a model host of infection. This elegant concept developed by Holden and colleagues was to allow a relatively rapid unbiased search for virulence genes using an animal host to select against strains carrying mutations in genes affecting virulence, among a mixed population of mutants. To avoid the typical labour-intensive screening of individual mutants in the first steps, each mutant is tagged with a different and unique DNA signature; the power of the STM technique allows large numbers of different strains to be screened at the same time in the same animal host.

In the classical STM protocol developed by Holden (3), a comparative hybridization technique is used and employs as mutagen a collection of transposons, each modified by the incorporation of a different DNA tag sequence. The concept here is that when the tagged mutagens integrate into the bacterial chromosome, each individual mutant can be distinguished from every single other mutant based on the unique and different tags carried by the strains. The first tag collection were originally designed as short DNA segments containing 40 bps variable regions flanked by invariant arms that facilitated the co-amplification and radioactive labelling of the central portions by PCR subsequently used as a hybridization probe (3).

Since STM is an *en masse* screening technique, mutagenized strains are usually kept individually in arrays using microtiter plates. Colony blots or DNA dot blots are prepared from this *in vitro* pool and compared by hybridization to the same pool of strains obtained, but after passage in the animal host. Technically, PCR is used to prepare labelled probes representing the tags present in the inoculum (input) and recovered from the host (output). Hybridization of the tags from the input and output pools to the colony or dot blot permits the identification of mutants that fail to grow *in vivo*, because these tags will not be present in the output pools. These strains can then be identified and recovered from the original arrays and the nucleotide sequence of DNA flanking the mutation site can be determined. Hence, STM has and will benefit immensely from the sequence data of all bacterial genomics projects.

In the original method, tags were incorporated into a miniTn*5* transposon and their suitability was checked prior to use by hybridization of amplified labelled tags to DNA colony blots used to generate the probes. Mutants whose tags failed to yield clear signals on autoradiograms were discarded; those that gave good signals were assembled into new pools for screening into animals (3). This careful analysis was done prior to STM so as to diminish the inherent problems of hybridization caused by problematic tags. The STM method was subsequently modified to avoid the pre-screening process where a series of Tn*917* transposons were selected prior to mutagenesis on the basis of efficient tag amplification and labelling, and lack of cross-hybridization to other tags (4). Since in this case the identity of each tag is known, each modified transposon can then be used to generate an infinite number of mutants, it can also be screened using plasmid or tag DNA rather than colony blots and increases the sensitivity and specificity of the assay. An additional modification of the technique was done where each of 96 tags were introduced at a disrupted *URA3* locus of *Candida glabrata* (5). For each tagged strain, different mutants were created by insertional mutagenesis using a plasmid that simultaneously complemented the *URA3* mutation. The DNA flanking the mutations could be cloned easily by plasmid rescue in *Escherichia coli*. From the original STM, several variations have been applied to many different bacterial pathogens on the basis of the particularities and limitations of each

living system studied with the data recently summarized (6). Indeed, the bacterial genetic tools of transposons are not easily amenable to genetic analysis in some species of bacteria. For example, Tn*5* transposons and their derivatives do not transpose well in some Gram negative bacteria such as *Pasteurellacea*, while not at all in Gram positive bacteria. However, the recent development of new transposons as tools for genetic analysis in a wide range of organisms and development of a complete *in vitro* transposition assay should permit the application of STM to a large variety of life forms (7). We present here a general approach of PCR-based STM but that can be adapted to any organism of interest.

General Strategy for the PCR-based STM

As in the original STM, PCR-based STM is divided into two major steps, an *in vitro* step and the *in vivo* selection step (Fig1 A and Fig 1 B). The first step involves the construction of specific and defined DNA tags and the preparation of the library of tagged mutants for the organism to be studied. In this first phase, a strategy needs to be devised and careful planning implies: a) the design of DNA tags as oligonucleotides; b) the cloning of these tags into a transposable element that gives reliable mutations in the organism to be studied by insertional mutagenesis; c) the transfer of the transposon containing specific tags to the organism to be analyzed and a reliable method to select the recipients; d) the assembly of an array of tagged mutants. The second major step necessitates an animal or presumably a cell model for *in vivo* screening of the library. In this second phase, a strategy needs to be defined so as to consider the power and the limitations of the animal models used and decide the number of rounds of STM screening and the number of animals to be utilized. Also, STM involves a systematic characterization of mutant strains selected *in vivo*. The first crucial data is: 1) to obtain as much DNA sequence as possible around the site of the insertion mutation by cloning the transposon marker or by RT-PCR; 2) to confirm that the tagged mutation is the cause of virulence attenuation. The final phase in STM is to classify

the genes identified as playing a significant role in virulence, define their function and their role in pathogenesis. Indeed this is the ultimate goal of STM. Here, we describe the rationale and step by step protocols essential for PCR-based STM and demonstrate typical results using *P. aeruginosa* as a model organism to be studied.

Methods

The design of oligonucleotides as signature DNA tags

A collection of complementary oligomers are first synthesized, rendered double stranded DNA, and are cloned into a mobile element for insertional mutagenesis, in this case derivatives of transposon mini-Tn*5*s. The PCR-based STM method that we developed involves designing pairs (24 in this case, but in theory 48 and 96 could be utilized) of 21-mers (Table 1) synthesized as complementary DNA strands for cloning into the mini-Tn*5*Km2, miniTn*5*Tc and miniTn5TcGFP plasmid vectors. The rationale here was to limit the tag complexity and used the power of bacterial genetics phenotypic selection to obtain a collection of 72 mutants per pool (selected using the miniTn*5*markers Km, Tc, Tc or GFP (Fig 3). There is a precedent for limiting the number of tags used. When *Salmonella typhimurium* was inoculated into the peritoneal cavities of mice, pools of 96 different mutants gave reproducible hybridization signals after 3 days of infection, whereas 192 did not (3). Problems with the complexity of the pool tags has been described also for *Vibrio cholerae* where it was necessary to reduce the complexity of orally inoculated pool to 48 strains to give reproducible results (8).

The sets of 24 tags are repeatedly used to construct 24 libraries (Fig. 1 A) and used for specific DNA amplifications as signature tags easily detectable by multiplex PCR (Fig. 4). Tagged products from arrayed bacterial clones can be compared as DNA products of a specific length separated by agarose gel electrophoresis.

Protocol 1: Synthesis and in vitro construction of tagged plasmids.

Twenty-four pairs of 21-mers were used. The first twelve pairs corresponded to ones described in Lehoux *et al.*, 1999 (9) and the other twelve pairs were designed following the same three basic rules: *(i)* similar T_m of 64°C to simplify tag comparisons by using one step of PCR; *(ii)* invariable 5'-ends with higher ΔG than at the 3'-end to optimize PCR amplification reactions; *(iii)* a variable 3'-end for an optimized yield of specific amplification product from each tag.

Annealing of complementary DNA:

1. A collection of twenty four defined 21-mers oligonucleotides were synthesized along with their complementary DNA strands as tags and are listed in Table 1). The synthesis was supplied by MGW Biotech. Inc. (High Point, NC, USA).

2. Annealing reactions contained 50 pmoles of both complementary oligonucleotides in 100 µl of medium salt buffer.

3. This oligonucleotide mixture is heated 5 min. at 95°C, left to cool slowly at room temperature in the block heater, and kept on ice.

Cloning of tags into a transposable element

The cloning if tags into an appropriate transposable will depend upon the organism to be studied by insertional mutagenesis. A plethora of mobile elements are currently available (7). The number of transposons that can be used in STM is limited by several factors including easy manipulation *in vitro* for DNA constructions, high frequency of transposition and random insertion in the genome of the host and controlled frequency of insertions as in miniTn*5*s. Obviously, STM cannot provide any information about genes that are essential which are totally inactivated by insertional mutagenesis. In contrast, an insertion within a non-essential gene crucial in virulence would not be expected to affect the growth rate of cells (10).

In our case for *P. aeruginosa*, each of the 3 pUTmini-Tn*5* plasmid DNAs (pUTmini-Tn5Km2; pUTmini-Tn*5*Tet and pUTmini-Tn*5*TetGFP) was used (12, 13, 14). This collection of Tn5-derived minitransposons has been constructed that simplifies substantially the generation of insertion mutants, *in vivo* fusions with reporter genes, and the introduction of foreign DNA fragments into the chromosome of a variety of Gram negative bacteria. The miniTn*5* consists of genes specifying resistance to kanamycin, tetracycline and the green fluorescent protein (Fig. 2) with unique cloning sites for tag insertion flanked by 19-base-pair terminal repeats, the I and the O ends. The transposons are located on a R6K-based suicide delivery plasmid pUT where the Pi protein is furnished by the donor cell; the pUT plasmid provides the IS50R transposase *tnp* gene in *cis* but external to the mobile element and whose conjugal transfer to recipients is mediated by RP4 mobilization functions in the donor (15).

Plasmid DNA was prepared, ligated with double stranded DNA tags in 24 separate reactions (16). The pUTmini-Tn*5* Km2 was digested with *Kpn*I (New England Biolabs, Mississauga, Ontario, Ca.) and recombinant molecules constructed *in vitro* by blunt-end fill-in with T4 DNA polymerase (GIBCO BRL Products, Gaithersburg, MD, USA). The pUTmini-Tn*5* Tc and Gfp were digested with *Not*I (New England Biolabs, Mississauga, Ontario, Ca.) and recombinant molecules constructed *in vitro* by blunt-end fill-in with Klenow (New England Biolabs). The ligation reactions were done using T4 DNA ligase, following the manufacturer's recommendations. Plasmids were transformed into *E. coli* S17-1λ*pir* (15) by electroporation and transformants were selected on TSA (Difco, Detroit, MI, USA) supplemented with 50μg/ml of ampicillin (Ap) (Sigma Chemical, St. Louis, MO, USA) and kanamycin (Km) (Sigma Chemical) for pUTmini-Tn*5* Km2, tetracycline (5 μg/ml) for pUTmini-Tn*5* Tc and Gfp. Technically, we provide below a simple protocol for ligation, cloning and electroporation.

Protocol 2: Ligation of tags, electroporation and screening of mini-Tn5s

1. 0.04 pmoles of plasmid are ligated to 1 pmole of double stranded DNA tags in a final volume of 10 µl of T_4 DNA ligase 1X buffer containing 400 units of T_4 DNA ligase.

2. Ligated products are purified using microcon PCR (Millipore) as described by the manufacturer's instructions and resuspended in 5 µl of H_2O.

3. All the 5 µl containing ligated products are transformed into *E. coli* S17-λ*pir* by electroporation using a Bio-Rad apparatus at 2.5 KV, 200 Ohms, 25 µF in a 2 mm electroporation gap cuvette. After electroporation, 0.8 ml of SOC is added to cells which are transferred in culture tubes to incubated 1 hour at 37°C.

4. Transformed bacteria containing tagged plasmids are selected on TSB supplemented with 50 µg/ml of ampicillin and 50 µg/ml of kanamycin by plating 100 µl of transformed cells.

5. Single colonies are selected, purified and screened by colony PCR in 50 µl reaction volumes containing: 10 µl of boiled bacterial colonies in 100 µl of TE PCR (10 mM Tris-HCl pH 7.4; EDTA 0.1 mM); 5 µl of 10X *Taq* polymerase (GibcoBRL) reaction buffer; 1,5 mM $MgCl_2$; 200 µM of each dNTPs; 10 pmoles of one of the oligonucleotide tag (9) used to construct the DNA tags as a 5-' primer and 10 pmoles of the pUTKanaR1 (5'-GCGGCCTCGAGCAAGACGTTT-'3) as the 3-' primer in the kanamycin resistance gene; 2,5 units of *Taq* polymerase (GibcoBRL). Thermal cycling conditions were (touchdown PCR): a hot start for 7 min. at 95°C, 2 cycles at 95°C for 1 min., 70 to 60°C for 1 min., and at 72°C for 1 min., then followed by 10 cycles at 95°C for 1 min., 60°C for 1 min., 72°C for 1 min. in a DNA Thermal Cycler (Perkin Elmer Cetus). Ten microliters of DNA amplified products were analyzed by electrophoresis in a 1% agarose gel, 1X Tris-borate EDTA buffer and stained for 10 min in 0.5 µg/ml ethidium bromide solution (16). The amplified product will have a size of 500 base pairs.

Protocol 3: Growth of bacterial strains

Escherichia coli strains were grown in tryptic soy broth (TSB). *P. aeruginosa* strains (PAO1 and PAO909) were grown in brain heart infusion (Difco). When needed, these media were supplemented with 1.5% of bacto-agar, ampicillin (50 µg/ml), chloramphenicol (5 µg/ml), kanamycin (50 µg/ml for *E. coli* and 500 µg/ml for *P. aeruginosa* in media with Bacto agar, and 300 µg/ml in liquid media), tetracycline (5 µg/ml for *E. coli* and 15 µg/ml for *P. aeruginosa*))

Transfer of the transposon containing specific tags to the organism to be analyzed

The signature tags inserted into transposons are replicated into *E. coli* and will need to be inserted into the chromosome of the host to be studied. With bacteria, this is usually achieved by conjugation at a high frequency. Generalities on the methods of transfer that can be used in any system of choice will depend upon the simplicity and capability of transferring the tags to the organism to be studied. We present below the protocol used with *P. aeruginosa* where transfer by mating is at a high level.

Protocol 4: Construction of 72 mini-Tn5 P. aeruginosa mutant libraries

1. *E. coli* S17-λ*pir* containing the pUTminiTn*5* plasmids with tags is used as a donor for conjugal transfer into the recipient strain, in our case *P. aeruginosa* PAO1. It is important to establish the ratio of donor: recipient to obtain the maximum of exconjugants (17). For *P. aeruginosa*, we used 1 donor for 10 recipient cells. Bacterial cells are mixed and spotted as a 50 µl drop on a sterile nylon membrane placed on a non-selective BHIA plate. Plates are incubated at 30°C for 8h.

2. Filters are washed with 10 ml of sterile phosphate buffered saline to recover bacteria.

3. Five 100 µl aliquots of the PBS solution containing exconjugants are plated on 5 BHIA plates supplemented with the appropriate antibiotics to select for the strain. Kanamycin is used to select exconjugants with the mini-Tn*5*Km2 inserted into their chromosomes and plates are incubated overnight at 37°C.

4. Selected colonies are picked on BHIA supplemented with ampicillin to exclude bacterial colonies having the suicide donor plasmid pUTmini-Tn*5* Km2 inserted into the chromosome by homologous recombination. Exconjugants were selected on BHIA supplemented with Cm (5µg/ml) (Sigma Chemical) and Km (500 µg/ml) for the mini-Tn*5* Km2 or Tc (15 µg) for the mini-Tn*5* Tc and mini-Tn5*gfp*.

5. Kanamycin resistant and ampicillin sensitive exconjugants are arrayed as libraries of 96 clones in 2 ml 96-wells plate in 1.5 ml of BHI supplemented with kanamycin and appropriate antibiotic.

6. As an STM working scheme, one mutant from each library is picked to form 96 pools of 72 unique tagged mutants in wells of 2 ml microtiter plates. The 2 ml 96-wells plates are incubated from 18-22h at 37°C.

Assembly of an array of tagged mutants

The exconjugants were arrayed as libraries of 96 clones in 2 ml 96 wells plates. In a defined library, each mutant had the same tag but assumed to be inserted at a different location in the bacterial chromosome. As an STM working scheme, one mutant from each library was picked to form 96 pools of 72 unique tagged mutants.

Screening of STM mutants in animal models

STM usually implies the use of an animal or simple cell model to mimic an insect, a plant, an animal or a human disease. As expected, these models cannot necessarily reproduce faithfully all the conditions of the infection. Several parameters need to be considered: 1) the number of different mutant strains to be used in a pool; 2) the route of administration, the dose for the inoculum and the incubation period; 3) an additional consideration is the use of different animal hosts for screening the same pool of STM mutants.

The inoculum size necessary to initiate an infection will determine the complexity of mutants pooled. In fact, each mutant in a defined input pool has to be in a sufficient cell number to initiate infection. The inoculum size must not be too high, resulting in the growth of mutants which would otherwise have not been detected. At higher doses, the immune system may be overwhelmed and the animals die of shock (6). Other important parameters in STM include the route of inoculation and the time-course of a particular infection. Also, certain gene products important directly or indirectly for initiation or maintenance of the infection may be niche-dependent or expressed specifically in certain animal or plant tissues only. If the duration of the infection in STM *in vivo* selection is short, genes important for establishment of the infection will be found, and if the duration is long, genes important for maintenance of infection will be identified (6). As STM is used and parameters are better defined in different models of infection, several routes of inoculation and different animal models can be used for the same organism studied by STM. We present below the protocol that we have used with *P. aeruginosa*.

Protocol 5: Chronic infection in the rat lung

For *P. aeruginosa*, we have used the chronic lung infection in a rat model which was adapted for this work (18). Female Sprague-Dawley rats of 140 to 160 g in weight were used. Isofluorane anesthetized rats were inoculated into the left lobe of lungs with 100 µl of a suspension of agar beads containing 10^6 bacterial cells (the *in vitro* pool). After 7 days, rats were sacrificed and lungs were removed and homogenized tissues were plated on BHIA supplemented with chloramphenicol. A concentration of 10^4 colonies was recovered after *in vivo* selection and was used for colony PCR (the *in vivo* pool) as described previously (6). Ninety-six pools of 72 mutants forming a collection of 6912 mutants were maintained in the rat animal model causing a chronic lung infection. We used an infecting dose of 10^6 bacteria per animal to ensure an initial inoculum of 10^5. After 7 days of infection, the lungs were isolated and an average of 10^6 bacteria was recovered from the organ of each animal. To identify mutants not recovered after the *in vivo* passage, screening was done by PCR using bacterial colonies. Mutants which gave no amplification products by multiplex PCR after the *in vivo* selection were retested by single PCR. Colony PCR amplification products obtained from the *in vitro* pool was compared to the *in vivo* pool.

Mutants which gave positive results from the *in vitro* pool and absent from the *in vivo* pool were kept for further analysis (Fig. 3). From 6912 *P. aeruginosa* mutants tested, we identified 214 attenuated mutants whose tag did not give a PCR amplification product from the *in vivo* pool. Attenuated mutants were assessed for growth on minimal media (M9 plates); 5 auxotrophic mutants were identified.

Protocol 6: Screening of tagged mutants by multiplex PCR

Detection of mutants was done by doing multiplex colony PCR in 50µl reaction volumes containing:

1.10 µl of boiled bacterial colony in 100 µl of TE PCR (10 mM Tris pH 7,4; 0,1 mM EDTA); 5 µl of 10X HotStart *Taq* polymerase (Qiagen) reaction buffer containing 15 mM $MgCl_2$; 200 µM of each dNTPs; 10 pmoles of one of 21-mers.

2. The 21-mers numbered 1 to 24 in Table 1 were used as a first primer in combination with 10 pmoles of pUTKana2, 10 pmoles of tetR1 primer and 10 pmoles of pUTgfpR2 primer. HotStart *Taq* polymerase 2.5 U (Qiagen, Mississauga, Ont., Canada) was used in each PCR.

3. Amplification conditions were: hot start 15 min. at 95°C, 2 cycles at 95°C for 1 min., 65 to 55°C for 1 min., and at 72°C for 1 min. followed by 10 cycles at 95°C for 1 min., 55°C for 1 min., 72°C for 1 min. (touchdown PCR) in a ICycler (BioRad).

4. Amplified products were analyzed in a 1% agarose gel, 1X Tris-borate EDTA buffer and stained in 0.5µg/ml ethidium bromide solution.

5. PCR amplification products of tags absent in the *in vivo* pool are compared with amplified products of tags present in the *in vitro* pool (Fig. 3).

6. Mutants that give PCR amplification products from *in vitro* pool and not from *in vivo* pool are purified and kept for further analysis.

7. To confirm STM mutants, the PCR reaction is repeated for each individual STM mutant giving a negative amplification product in the multiplex PCR screening step. These STM mutants are carefully identified and used for a second round of *in vivo* screening.

Second round of *in vivo* screening

We arrayed 14 new groups with the 214 mutants from the first screening and 29 *P. aeruginosa* STM mutants previously screened once to confirm the STM attenuated phenotype (19, 20). When necessary, we have completed a group with the wild-type strain PAO1 so as to always maintain 72 clones in each group. The *in vivo* selection and detection of mutants were done as described above. From the 214 mutants initially identified, we retained 42 highly attenuated mutants whose tags did not give any PCR amplification product from the *in vivo* pool. These results showed that to identify and obtain the most significant and highly attenuated mutants, a second round of *in vivo* screening is a pre-requisite. The genomic DNA from these *P. aruginosa* STM mutants was isolated, digested with *Ps*tI and cloned into pTZ18R (Fig. 4)

Protocol 7: Cloning and analysis of disrupted STM genes mutants selected.

1. Chromosomal DNA from the STM mutants selected is prepared using the QIAGEN genomic DNA extraction kit as described in the manufacturer's protocol.

2. Chromosomal DNA (1µg) is digested with a restriction endonuclease giving a large range of fragment sizes; in our case we utilized *PstI* with *P. aeruginosa* and cloned DNA fragments ranging in size from 1 Kb to 6 Kb.

3. Digested chromosomal DNAs are cloned into pTZ18R (Amersham Pharmacia Biotech) predigested with the corresponding restriction enzyme. Ligation reactions are done as follows: 1 µg of digested chromosomal DNA is mixed with 50 ng of digested pTZ18r in 20 µl of 1X T_4 DNA ligase buffer with 40 units of T_4 DNA ligase. Incubate overnight at 16°C.

4. DNA ligation products are purified using the microcon PCR (Millipore) as described by the manufacturer's instructions and the DNA is resuspended in 5 µl of H_2O.

5. The 5 µl recombinant plasmid solution is used for electroporation into *E. coli* DH5α.

6. Bacterial clones are kept and purified for plasmid analysis.

7. Plasmid DNA is prepared with QIAGEN midi preparation kit as described by the manufacturer.

Characterization of mutant strains

When a potential STM mutant has been obtained, it is essential to rapidly confirm that the tagged mutant is the cause of attenuation, even after two rounds of *in vivo* screening. Our recent approach is to do this first, even before the actual DNA sequence flanking the insertion point is known. The degree of virulence attenuation is a pre-requisite and we use the more sensitive and increasingly popular competitive index (CI) tests (21). The analysis is done with an STM mutant strain combined as single or mixed infections with the wild-type parent strain to describe the kinetics of growth, and in certain cases to identify the time and body site where the virulence defect is apparent. In a mixed infection, the ability of the strains to initiate or colonize the host provides a measure of their relative virulence. The CI can then be defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria. The CI is thus a quantitative value for the degree of attenuation of a mutant strain, with the CI of a wild-type strain versus a fully virulent derivative being approximately 1.0 (21).

Protocol 8: CI of P. aeruginosa STM mutants

The *in vitro* growth rates of the PAO1 and mutant strains were measured in the chronic rat lung model. Here, we present a variation of the CI, the growth index.

1. A collection of bacterial strains (PAO1 wild-type, PAO909, a Pur- mutant and STM mutants isolated *in vivo*) were pre-cultured overnight in 10 ml of BHI adding kanamycin in the case of the mutants.

2. These cultures were diluted 1:100 in fresh medium. At different time points, aliquots of cultures were diluted and plated on BHIA to determine colony-forming units (CFU) per ml. The growth indices are given by the ratio of growth rate during exponential growth of the mutant on the ratio of the wild-type strain.

3. For the *in vivo* growth rates, both strains were pre-cultured overnight in 10 ml of BHI with kanamycin in the case of the mutants. From the pre-culture, 1 ml was washed 3 times in PBS, and 100 µl of washed pre-cultures were used for preparation and inoculation of agar beads as described above.

4. Bacteria were recovered after one and seven days post-infection from 2 animals for each point by direct plating of lung tissue homogenates on BHIA plates supplemented with chloramphenicol (5 µg/ml). Animals used as controls were injected with PAO909 strain (a purine auxotroph of PAO1) and PAO1.

5. Nine mutants were found to be attenuated and are listed in Table 2. Among the nine *in vivo* attenuated mutants, levels of growth rate varied from low to high as reflected by index values the selected mutants obtained (Table 2). The less attenuated mutant has an index of 0.6, taking into consideration that this mutant is slightly attenuated *in vivo*. Mutants that were considered to be clearly attenuated had a growth rate index of 0.001.

An additional confirmation that we use in combination with the CI is to show that the tagged mutation is the cause of attenuation of virulence is by complementation with a functional allele. In this case, the wild-type gene of the STM mutant identified is cloned as a PCR product and introduced in the STM mutant. In a first step, we use RT-PCR to demonstrate *in vitro* expression and proceed with a CI analysis of the wild-type, the STM mutant and the mutant strain expressing the complementing allele. The obvious result expected will show that the mutation and the virulence phenotype are linked. Gene analysis

can indicate if the inactivated gene is part of an operon and that STM attenuation of virulence may be due to a polar effect. In this case, inactivation of the downstream gene(s) is essential to confirm their implication in the decrease of virulence by further testing with the CI tests.

To demonstrate allelic complementation of STM mutants, we will use the G18T12 mutant described in Tables 1and 2 (20). Mutant G18T2 corresponds to an insertion in an ORF (PA0158) encoding for a protein of 1016 residues and having 32% identity with a putative ORF VC1673 of *Vibrio cholerae*, 31% identity with AcrD of *Rickettsia prowazekii* and CzrA of *P. aeruginosa* and 45 % identity with a membrane protein which is a putative cation efflux protein (HP0969) of *Helicobacter pylori* also identified as Hef. The protein PA0158 has been classified as a probable multidrug efflux system component encoding for a probable RND efflux transporter. In *H. pylori*, *hefF* encodes an RND pump protein homolog. The role and function of RND multiple-drug efflux systems in intrinsic antibiotic resistance has been demonstrated in several human pathogens, including *E. coli*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *P. aeruginosa*. The corresponding mutant has been shown to be mildly attenuated with a GI value of 0.294 in Table 2. Several pseudomonads possess an intrinsic resistance to many front-line antibiotics, due mainly to its low outer membrane permeability and to the mechanism of active efflux of antibiotics. We have found by STM that this efflux system may be implicated *in vivo*; this system could presumably be active against the host defense system by pumping out toxic molecules.

As proof of concept, we have cloned, and expressed the *modA* gene, as determined by RT-PCR (Fig. 5) and shown complementation of ModA *in vitro* and *in vivo* using the mutant G13T12 (PA1863). The complemented strain G13T12/pMON-PA1863 was used and compared to the G13T12 strain. We observed that the complemented strain was able to grow 3.4 times faster *in vivo* than the strain containing the *modA* insertional mutation (GI of 0.24 compare to 0.07, respectively). These results showed that the complementation with

modA gave a significant recovery to the wild-type phenotype. This indicated that the disruption of *modA* in the mutant G13T12 was responsible for the attenuated phenotype obtained by STM, and confirmed by the *in vivo* GI experiments. Indeed, insertional inactivation of *modA* gave a significant attenuation of virulence *in vivo* but attempts to restore to the wild-type phenotype the mutant G13T12 was not complete and partly successful (GI of 0.6 instead of 1.0).

Protocol 9: preparation of RNA for RT-PCR

Total RNA from *P. aeruginosa* wild type strain, the modA mutant and the complemented mutant were obtained by RNeasy Midi Kit (Qiagen) flowing recommended procedure by Qiagen. All preparations were done using Aerosol-Resistant Tips and glove under a fume hood.

Protocol 10: DNA removal from RNA preparation

The total RNA preparation was treated with DNaseI prior to RT-PCR analysis. Careful removal of any remaining DNA in the RNA preparation is crucial and appropriate controls should be done to confirm that no DNA is present.

1. The DNase procedure was done using RNase-Free DNase Set (Qiagen) prepared folllowing the recommended procedures by Qiagen. Solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water was prepared. The reagent is kept in aliquots of 10 µl in microtubes at -20°C, this stock is stable up to 9 months.

2. DNase digestion:

100 µl RNA (80 µg)

10.3 µl Buffer RDD

2.9 µl DNase I stock solution (7.8 Kunitz)

3. Incubation 1h at 37°C.

4. Heat inactivation of DNase I 15 min. at 75°C. Repeat this DNase I digestion if necessary (if the PCR control is still positive with DNA).

Protocol 11: PCR confirmation for absence of DNA in the RNA preparations

The absence of DNA in each RNA preparation was confirmed by PCR using the following protocol. PCR reactions were done with the primer that will be used in RT-PCR to amplify the gene *mod*A (PA1863) used for complementation (RT1863FOR 5'- CCG ATC CAG GCC ATC GCC AAG -3', RT1863REV 5'- CGA CAG AGC GAC GAA GCC CAG -3') and primers used for controls from the *fts*Z gene (ftsZ3 5'- CAT CGC ACA AAC GCG CGT CAT -3', ftsZ4 5'- ACG CAG GAA CGC CGG GAT ATC -3').

1. Mix 10 µl of RNA preparation (1.4 µg), 5 µl of 10x HotStartTaq PCR buffer (Qiagen) (1.5 mM $MgCl_2$), 8 µl dNTP (200 µM), 2 µl of each primers (0.1 µM) (RT1863FOR-RT1863REV pairs or ftsZ3-ftsZ4 pairs), 0.5 µl HotStartTaq DNA polymerase (2.5 U) (Qiagen) and complete the final volume up to 50 µl with nanopure water.

2. Reactions were performed in 200 µl thin-wall microtube in the Icycler thermocycler (BioRad).

3. HotStartTaq DNA polymerase requires an activation step of 15 min. at 95°C.

4. Amplification was done using TouchDown cycles: 1 min. at 94°C denaturation, 1 min. at 70°C annealing, 1 min. at 72°C elongation, every 2 cycles annealing temperature decreased by 1°C, 22 cycles were done until annealing temperature reach 60°C.

5. Amplification continued for 10 cycles: 1 min. at 94°C denaturation, 1 min. at 60°C annealing, 1 min. at 72°C elongation. A final step of 7 min. at 72°C was done to complete elongation of PCR products.

6. DNA amplification products were visualized by agarose gel electrophoresis and positive reactions indicates the presence of DNA contamination. DNase I digestion is repeated and the preparation is tested by PCR.

Protocol 12: RT-PCR reactions

The RT-PCR was done on treated RNA from *P. aeruginosa* wild type strain, the mutant and the complemented mutant using internal primers from the *mod*A sequence (RT1863FOR 5'- CCG ATC CAG GCC ATC GCC AAG -3', RT1863REV 5'- CGA CAG AGC GAC GAA GCC CAG -3'). The expected result is to confirm transcription of *mod*A for positive complementation as visualized in an agarose gel (Fig.5).

1. Mix 10 µl of the RNA preparation (1.4 µg), 10 µl of 5x OneStep RT-PCR buffer (Qiagen) (1.25 mM $MgCl_2$), 2 µl dNTP (400 µM), 6 µl of each primers (0.6 µM) (RT1863FOR-RT1863REV), 2 µl OneStep RT-PCR Enzyme Mix (Qiagen) (containing Omniscript Reverse Transcriptase, Sensiscript Transcriptase and HotStartTaq DNA polymerase) and raise final volume up to 50 µl with RNase-free water water.

2. Reactions are performed in 200 µl thin-wall microtube in Icycler thermocycler (BioRad).

3. Reverse Transcription is done in the thermocycler 30 min. at 50°C.

4. HotStartTaq DNA polymerase requires an activation step of 15 min. at 95°C, Omniscript and Sensiscript Reverse Transcriptases were inactived at the same time.

5. Amplification was done using TouchDown cycles: 1 min. at 94°C denaturation, 1 min. at 70°C annealing, 1 min. at 72°C elongation, every 2 cycles annealing temperature decrease by 1°C, 22 cycles were done until annealing temperature reach 60°C.

6. Amplification was continued for 10 cycles at: 1 min. at 94°C denaturation, 1 min. at 60°C annealing, 1 min. at 72°C elongation. A final step 7 min. at 72°C was done to complete PCR product.

7. Amplifications were visualized on agarose gel electrophoresis and positive reaction indicated RNA transcription of *mod*A.

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Table 1. Nucleotide sequences of signature tags used in PCR-based STM.

Tag number	Nucleotide sequence
1	GTACCGCGCTTAAACGTTCAG
2	GTACCGCGCTTAAATAGCCTG
3	GTACCGCGCTTAAAAGTCTCG
4	GTACCGCGCTTAATAACGTGG
5	GTACCGCGCTTAAACTGGTAG
6	GTACCGCGCTTAAGCATGTTG
7	GTACCGCGCTTAATGTAACCG
8	GTACCGCGCTTAAAATCTCGG
9	GTACCGCGCTTAATAGGCAAG
10	GTACCGCGCTTAACAATCGTG
11	GTACCGCGCTTAATCAAGACG
12	GTACCGCGCTTAACTAGTAGG
13	CTTGCGGCGTATTACGTTCAG
14	CTTGCGGCGTATTATAGCCTG
15	CTTGCGGCGTATTAAGTCTCG
16	CTTGCGGCGTATTTAACGTGG
17	CTTGCGGCGTATTACTGGTAG
18	CTTGCGGCGTATTGCATGTTG
19	CTTGCGGCGTATTTGTAACCG

20	CTTGCGGCGTATTAATCTCGG
21	CTTGCGGCGTATTTAGGCAAG
22	CTTGCGGCGTATTCAATCGTG
23	CTTGCGGCGTATTTCAAGACG
24	CTTGCGGCGTATTCTAGTAGG

Each 21-mers has a T_m of 64°C and permits PCR amplification in one step when the 3 primer combinations are used for multiplex screening. Two sets of consensus 5'-ends comprising the first 13 nucleotides have higher ΔGs for optimizing PCR. Twelve variable 3'-ends define tag specificity and allow amplification of specific DNA fragments. The set of 24 21-mers representing the complementary DNA strand in each tag are not represented and can be deduced from the sequences present.

Table 2. *In vitro* and *in vivo* growth growth index of *P. aeruginosa* STM mutants.

Strains/(Genes inactivated)	*In vitro GI*[a]	*Relative GI*[b]
PAO1	1.0	1.0
PAO909	1.1	0.0036
G20T2 (Clp protease, ClpB, *Y. enterocolitica*)	1.5	0.0006
G13T12 (Molybdate, binding precursor, ModA, *P. aeruginosa*)	1.2	0.025
G19T12 (Unknown)	2.0	0.01
G10T7 (Unknown)	1.7	0.176
G30T12 (Hypothetical 64 kDa protein, YfaA, *E. coli*)	1.3	0.154
G56T2 (Hypothetical 51 kDa protein,	2.4	0.25

YgdH, *E. coli*)		
G18T2 (Transporter protein, AcrB, D, F, Vc1663, *V. cholerae*)	1.7	0.294
G94T2 (ABC transporter ATP,YbjZ, *E. coli*)	1.5	0.0267
G38T4 (Heat shock protein, HtpG, *P. aeruginosa*)	5.8	0.034

The disrupted open reading frames (ORF) in each STM mutant were: PAO090 (G20T2); PA1863 (G13T12); Pa5441 (G19T12); PAO082 (G10T7); PA4491 (G30T12); PA4115 (G56T2); PAO0158(G18T2); PA0073 (G94T2); PA1596(G38T4). Identity values were obtained by comparing the amino acid sequences deduced from the complete ORF.

[a] The *in vitro* growth index (GI) is the ratio of the growth rate of the mutant compared to that of the wild-type strain PAO1 in rich BHI broth.

[b] The relative growth index (GI) is the *in vivo* growth index divided by the *in vitro* growth index. PAO909 is a purine auxotrophic strain used as a negative control.

Figure Legends

Fig.1A.General strategy for the construction of the arrayed libraries tagged with miniTn*5*Km2, miniTn*5*Tc and miniTn*5*Tcgfp. In a defined library, each mutant has the same tag but inserted at different locations in the bacterial chromosome. One mutant from each library is picked to form 96 pools of 24 mutants with unique tag for each miniTn*5*.

Fig.1B. Comparative analysis between the *in vitro* and *in vivo* pools using multiplex PCR. An aliquot is kept as the *in vitro* pool, and a second aliquot from the same preparation is used for passage into an animal model for *in vivo* negative selection. After determined time points of infection, bacteria are recovered from animal organs and constitute the *in vivo* pool. The in vitro and in vivo pools of bacteria are used to prepare DNA in 24 PCR reactions using the 21-mers 1 to 24 in table 1 and the Km, Tc and gfp primers.

Fig.2. Physical and genetic maps of the miniTn*5*Km2, miniTn*5*Tet and miniTn*5*Tetgfp transposons used. The elements are represented by thick black lines, inverted repeats are indicated as vertical boxes and genes are indicated by arrows. Abbreviations: I and O inverted repeat ends; Km, kanamycin; Tc, tetracycline; gfp, green fluorescent protein.

Fig.3. Agarose gel electrophoresis separations of multiplex PCR DNA amplified products obtained from the *in vivo* pool of 72 tagged-transposon mutants. Lane 1 to 24, 10 µl of a multiplex PCR reaction using pUTKana2, tetR1 and pUTgfpR2 primers with Tag 1 to 24 respectively. The lane indicated as – is the negative control. The multiplex PCR conditions are described in the text. The amplified PCR products from tagged mini-Tn*5* Km2, *gfp* and Tc are 220, 820 and 980 bps respectively. Note the absence of amplification in lanes 5,7,15, 24 where one or two DNA bands are not visible. Rigorous analysis by PCR-based STM implies that the weak signals in lanes 2, 9,10,11,15, would be considered as positive amplification and would not be tested further. The clones in lanes 5, 7, 15 and 24 would be

further tested by individual PCR and an additional round of *in vivo* STM screening. In this specific example, no STM miniTn*5*Km mutants were obtained.

Fig.4. General scheme for cloning genomic DNA encoding the inactivated gene presumably implicated in attenuation of virulence via selection of the antibiotic resistance genes inserted in STM mutants. The genomic DNA of *P. aeruginosa* is digested completely with *Pst*I and cloned DNA fragments are selected on the basis of encoding either Tc or Km resistance. The cloned genomic DNA is sequenced using primers annealing in the I end of each transposon DNA fragment (I-End sequencing primer: 5'- AGA TCT GAT CAA GAG ACA G-3').

Fig. 5 Agarose gel electrophoresis of RT-PCR products confirming expression of modA from the complementing allele. Lanes: 1, wild-type; 2, modA STM mutant complemented; 3, STM mutant. The intense 500 bps band is due to high level expression from the *lacZ* constitutive promoter in pTZ18R.

Figure 1A

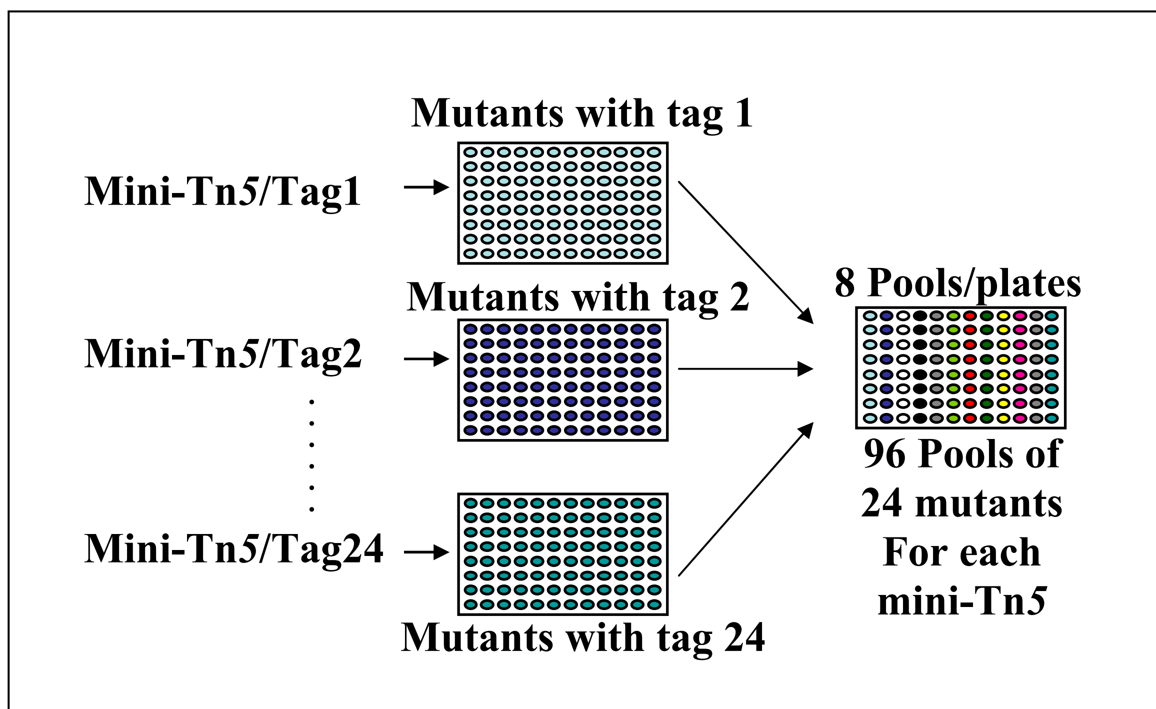


Figure 1B

96 mutants with specific tag

***In vitro* pool**

***In vivo* selection in animal model (*in vivo* pool)**

Tag1-24

I **O**

24 PCRs

1 7 24 **bps**
1000
820
650
220

vs

Tag1-24

I **O**

24 PCRs

1 7 24 **bps**
1000
820
650
220

Figure 2

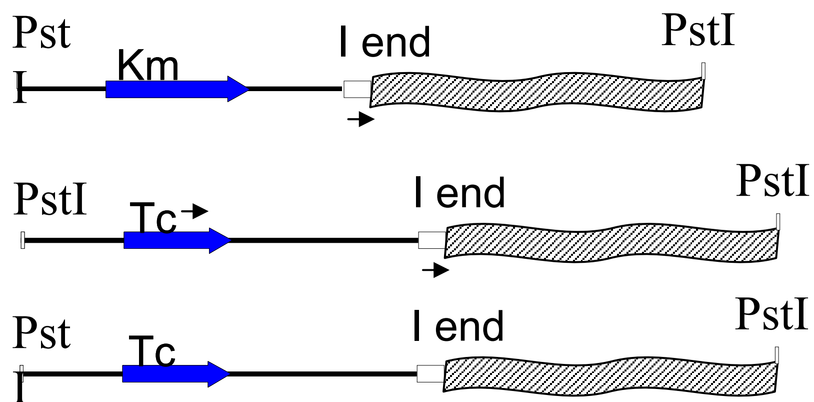


Figure 3

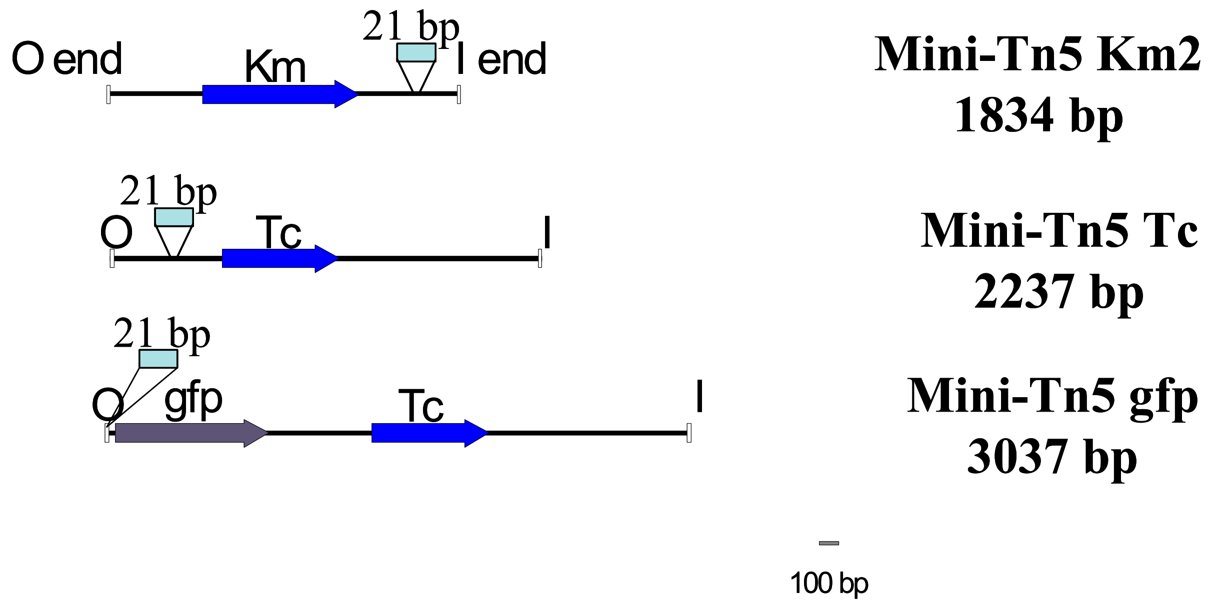


Figure 4

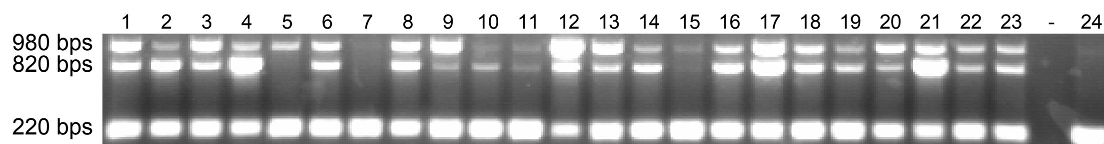
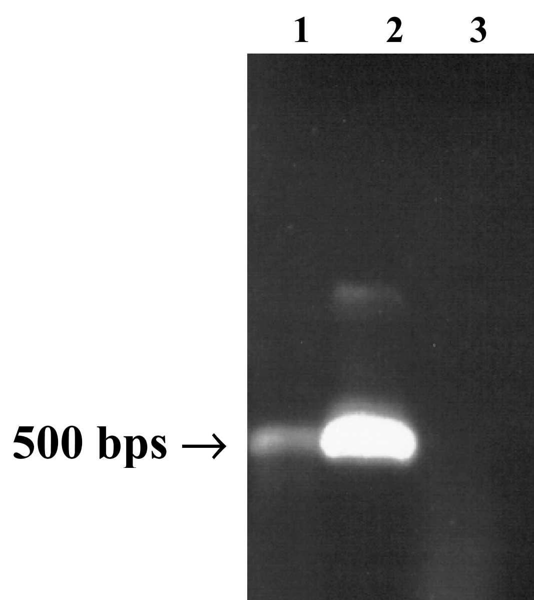


Figure 5



Appendice 2

Comparative Signature-Tagged Mutagenesis identifies Pseudomonas factors conferring resistance to the pulmonary collectin SP-A

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Comparative Signature-Tagged Mutagenesis Identifies *Pseudomonas* Factors Conferring Resistance to the Pulmonary Collectin SP-A

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The pulmonary collectin, surfactant protein A (SP-A), is a broad spectrum opsonin with microbicidal membrane permeabilization properties that plays a role in the innate immune response of the lung. However, the factors that govern SP-A's microbial specificity and the mechanisms by which it mediates membrane permeabilization and opsonization are not fully understood. In an effort to identify bacterial factors that confer susceptibility or resistance to SP-A, we used comparative signature-tagged mutagenesis to screen a library of 1,680 *Pseudomonas aeruginosa* mutants for evidence of differential pulmonary clearance in SP-A-sufficient (SP-A$^{+/+}$) and SP-A-deficient (SP-A$^{-/-}$) mice. Two SP-A-sensitive *P. aeruginosa* mutants harboring transposon insertions in genes required for salicylate biosynthesis *(pch)* and phosphoenolpyruvate-protein-phosphotransferase *(ptsP)* were recovered. The mutants were indistinguishable from the parental wild-type PA01 with regard to opsonization by SP-A, but they exhibited increased susceptibility to SP-A-mediated membrane permeabilization. These results suggest that bacterial gene functions that are required to maintain membrane integrity play crucial roles in resistance of *P. aeruginosa* to the permeabilizing effects of SP-A.

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Introduction

Pulmonary surfactant is a protein-phospholipid complex that lines the air-liquid interface of alveoli, forming the first point of contact with inhaled microbial pathogens [1,2]. A multitude of in vitro studies have demonstrated that the pulmonary collectins, surfactant protein A (SP-A) and surfactant protein D (SP-D), bind and aggregate bacterial, fungal, viral, and mycobacterial organisms, directly activate macrophages, and enhance the in vitro phagocytosis and intracellular killing of a variety of pulmonary pathogens [3,4]. Recognition of diverse microbial species by the pulmonary collectins is mediated by the C-type lectin domain, which selectively binds to carbohydrates that are prevalent on the surface of microbes, but not to the predominant terminal sugars on surface molecules of mammalian cells [5]. More recently, SP-A and SP-D have been reported to possess potent antimicrobial properties [6–8]. They directly inhibit the proliferation of bacteria and fungi in a macrophage- and aggregation-independent manner, by increasing the permeability of the microbial cell membrane. In particular, rough strains of Gram-negative bacteria, containing truncated lipopolysaccharide (LPS) domains are uniquely vulnerable to permeabilization by the collectins [6], as has been well known for other antimicrobial peptides and proteins.

Although the collectins inhibit microbial growth, the factors that govern the specificity and the mechanisms of membrane disruption by the collectins are not understood. In this study, we employed comparative signature-tagged mutagenesis (STM) as a tool to identify bacterial factors required to resist SP-A-mediated clearance, in vivo. STM relies on the ability of the pathogen in question to replicate in vivo as a mixed population and allows for the identification of the mutants whose phenotypes cannot be trans-complemented by other virulent strains present in the same inoculum [9]. Two *Pseudomonas* factors that are specifically required to protect *P. aeruginosa* from killing by SP-A were identified and characterized.

Results

SP-A$^{-/-}$ Mice Are Susceptible to Infection by *P. aeruginosa*

Levine et al [10] have reported that outbred SP-A$^{/}$ mice exhibit delayed clearance of a clinical *P. aeruginosa* strain from the lung, suggesting that SP-A plays a role in pulmonary innate immunity against this organism. We also found that the inbred C3H/HeN SP-A$^{/}$ mice were more susceptible to *P. aeruginosa* infection, in this case by strain PA01. SP-A$^{+/+}$ mice cleared 63-fold (1.8 log) more of intranasally inoculated PA01 by 16 h (Figure 1A). In contrast, the bacterial load increased by 22 fold (1.34 log) in SP-A$^{/}$ mice (Figure 1A). In the absence of infection, SP-A$^{/}$ mice are healthy, and the histopathological analyses of their non-infected lungs re-

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Abbreviations: CFU, colony forming unit; CI, competitive index; GFP, green fluorescence protein; LB, Luria broth; LPS, lipopolysaccharide; RFLP, restriction fragment length polymorphism; SP-A, surfactant protein A; SP-D, surfactant protein D; STM, signature-tagged mutagenesis; TLC, thin layer chromatography

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Synopsis

Everyday, normal breathing deposits numerous microorganisms on the surfactant membrane that lines the air-exchanging surfaces of the lung. Surfactant protein SP-A, a component of the surfactant membrane, helps to maintain the lung in a germ-free state by aggregating inhaled microorganisms and facilitating their ingestion by immune cells, and by increasing the permeability of their cell membranes. However, the bacterial pathogen *Pseudomonas aeruginosa* is resistant to SP-A-mediated membrane disruption.

Using a genetic tool called comparative signature-tagged mutagenesis, the authors have identified two *P. aeruginosa* genes, *pch* and *ptsP*, that are required to resist SP-A-mediated membrane permeabilization. Molecular analyses indicate that the *pch* gene encodes an enzyme that synthesizes salicylate, a compound utilized by bacteria to acquire essential metal ions. The *ptsP* gene encodes an enzyme called phosphoenolpyruvate-protein-phosphotransferase. The loss of salicylate and phosphoenolpyruvate-protein-phosphotransferase weakens the *P. aeruginosa* cell membrane, which allows SP-A to poke holes on the membrane and kill the bacteria.

This is the first known report of the roles played by salicylate and phosphoenolpyruvate-protein-phosphotransferase in maintenance of bacterial membrane, and consequently, protecting bacteria from killing by SP-A, through disruption of membrane integrity.

vealed no significant differences when compare to the lungs of SP-A$^{+/+}$ mice (unpublished data). When infected with PA01 however, SP-A$^{+/+}$ mice developed broncho-pneumonia with mild pulmonary infiltrates (Figure 1B). In contrast, SP-A$^{-/-}$ mice developed more extensive consolidation with areas of lobar pneumonia.

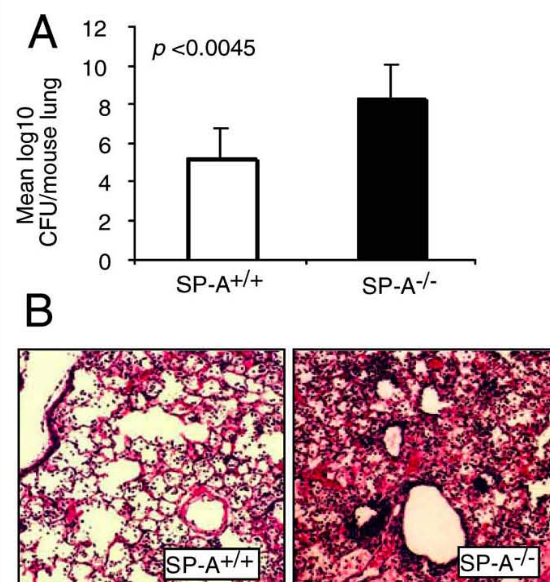

Figure 1. SP-A Deficient Mice Are More Susceptible to Infection by *P. aeruginosa*

(A) SP-A$^{+/+}$ and SP-A$^{-/-}$ mice (group of six) were infected intranasally with 1×10^7 *P. aeruginosa* PA01. Bacteria were recovered from homogenized lung tissue 16 h after inoculation. $p < 0.01$.

(B) Representative lung histology sections (stained with hematoxylin and eosin) from SP-A$^{+/+}$ and SP-A$^{-/-}$ mice 16 h post intranasal instillation of PA01. Original magnification: 10 ×.

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Identification of *P. aeruginosa* Genes Conferring Resistance to SP-A by Comparative STM Screening

We exploited the PCR-based STM technique and the *P. aeruginosa* PA01 STM mutant library [11] to identify putative microbial targets for SP-A. SP-A$^{+/+}$ and SP-A$^{-/-}$ mice, in groups of three, were intranasally challenged with pools of 72 oligo-tagged STM mutants. Lungs were harvested at 16 h, homogenized, and plated. At least 10,000 bacterial colonies grown on Luria broth (LB) agar plates were harvested from each lung for bacterial genomic DNA extractions and oligo-tag amplification. We screened for differential clearance of individual STM mutants using PCR. If the PCR product of a mutated gene was absent on the output gel in SP-A$^{+/+}$ mice, but present in SP-A$^{-/-}$ mice (Figure 2A, see arrows), we postulated that the bacterial protein encoded by the mutated gene was required to overcome the host defense activities of SP-A.

We comparatively screened 1,680 STM mutant strains in SP-A$^{+/+}$ and SP-A$^{-/-}$ mice. Two mutants with increased sensitivity to SP-A (i.e., failed to survive in SP-A$^{+/+}$ mice) were recovered, for a 0.12% recovery rate. We have cloned and sequenced both STM mutants by plasmid rescue [11]. DNA sequences were analyzed by comparison with online databases (www.pseudomonas.com). The output from Pool 4 (mutant *pch*, see below) is shown in Figure 2B (left panel). In the presence of SP-A, the *pch* mutant, which is disrupted in a gene required for salicylate biosynthesis, was unable to survive in the SP-A$^{+/+}$ lung (missing band in the box, Figure 2B). In absence of SP-A, the *pch* mutant was able to grow in the lung, as indicated by the presence of an oligo-tag amplified by PCR (Figure 2B, left panel, SP-A$^{-/-}$ lane; right panel, lane m1). The results were confirmed by PCR analysis of the output pool of two additional mice (Figure 2B, right panel, lanes m2 and m3).

The *pch* STM mutant carries a transposon pUTmini-Tn5Km2 insertion within an intergenic region in between the 10th and 11th nucleotide after the stop codon of the *pchA (PA4231)* gene (Figure 2C). The *pchA* gene encodes an isochorismate synthase that participates in the biosynthesis of the siderophores, salicylate, and pyochelin [12–14]. Because transposon inserted within the intergenic region immediately downstream of the *pchA* gene, it might have affected the mRNA stability of the *pchDCBA* transcript. Thus, we named the STM mutant as *pch*. We examined whether the *pch* mutant had lost the ability to synthesize pyochelin by using thin layer chromatography (TLC) assays. TLC analysis of pyochelin extracted from bacteria cultured in M63 minimal medium confirmed that the *pch* mutant is unable to synthesize the green-colored pyochelin (Figure 2D, *pch* lane). In contrast, the parental wild-type PA01 produced pyochelin (Figure 2D, PA01 lane). When the *pch* STM mutant was grown in the presence of 1 mM salicylate, however, the ability to synthesize pyochelin was restored (Figure 2D, *pch* + 1mM salicylate lane). The expression of the genes within the downstream operon *(PA4232* and *PA4233)*, which is transcribed in opposite orientation to the *pchADCBA* operon, was found to be unaffected by transposon insertion as assessed by

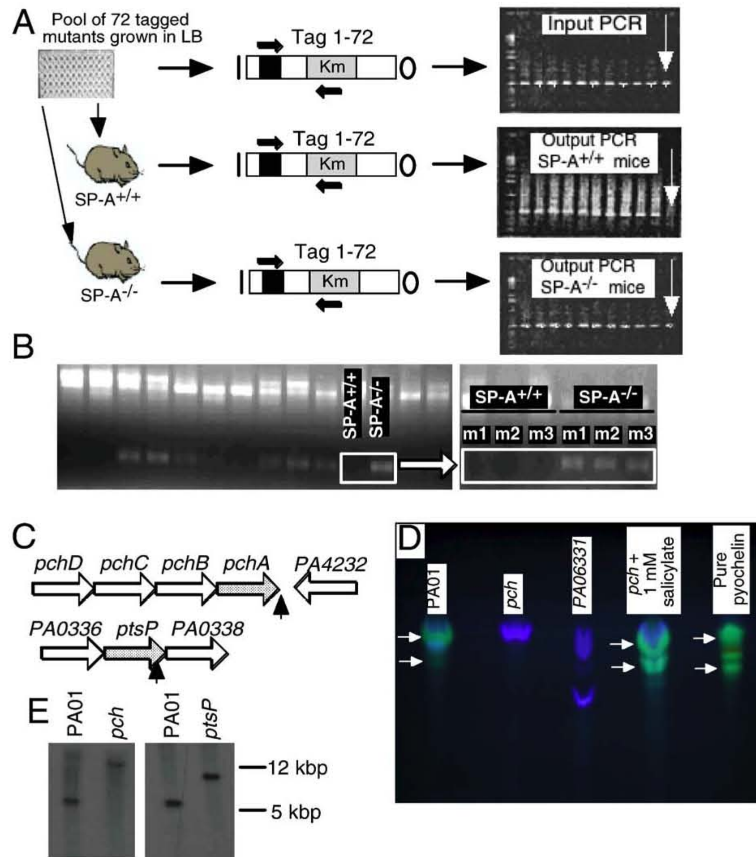

Figure 2. PCR-Based Signature-Tagged Mutagenesis

(A) Schematic drawing of PCR-based STM. Pools of 72 uniquely tagged mutants were intranasally inoculated into SP-A$^{+/+}$ and SP-A$^{-/-}$ mice; 16 h later, lungs were harvested, homogenized, and plated. Approximately 10,000 colonies were harvested from the plates for genomic DNA extraction. PCR-amplification of tags was performed on the genomic DNA to screen for the presence or absence of DNA tags of each of the 72 mutants. Mutants whose DNA tags were present in the input pool and in the SP-A$^{-/-}$ pool, but absent in the SP-A$^{+/+}$ pool (see white arrows) were further screened for susceptibility to SP-A.

(B) Agarose gel of PCR-based STM, identifying the *pch* mutant (left panel). Attenuation in the first SP-A$^{+/+}$ mouse (right panel, m1) was confirmed in two additional SP-A$^{+/+}$ mice (right panel, m2 and m3).

(C) Genetic loci of *P. aeruginosa*, when mutated, conferred increased sensitivity to in vivo killing by SP-A. DNA regions flanking pUTmini-Tn*5* transposon insertions were cloned and sequenced. Similarity BLAST searches were performed against *P. aeruginosa* PA01 genomic sequence on NCBI and on www.pseudomonas.com. Black arrows indicate the approximate insertion site within the mutated ORFS.

(D) TLC analyses indicate that the *pch* STM mutant is unable to synthesize pyochelin (see arrows). Wild-type PA01 grown in LB and the *pch* mutant grown in LB supplemented with 1 mM salicylate produced green color pyochelin (see arrows). In contrast, no observable pyochelin was produced by the *pch* and *PA06331* strains. Pure pyochelin was used as control.

(E) Restriction fragment length polymorphism analysis between parental wild-type PA01 and STM mutants confirmed that mutations in *pch* and *ptsP* were caused by a single insertion.

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mRNA expression analyses (unpublished data). Collectively, these data suggest that susceptibility to SP-A-mediated clearance is caused by the loss of *pch* gene function, most probably by affecting the mRNA stability of the *pchDCBA* operon.

The second SP-A-susceptible STM mutant had the transposon pUTminiTn*5*Km2 inserted in between the 2,213th and 2,214th nucleotides of the *ptsP (PA0337)* gene (Figure 2C). The *ptsP* gene encodes a phosphoenolpyruvate-protein-phosphotransferase, a homolog of the *E. coli* Enzyme I^{nitrogen} (EI^{Ntr}) [15]. The transposon pUTminiTn*5*Km2 insertion into *ptsP* is predicted to cause polarity and interrupt the transcription of *PA0338*.

Both STM mutants were judged to harbor a single transposon pUTminiTn*5*Km2 insertion after hybridization with the transposon vector (unpublished data). DNA fragments that flanked both ends of the transposon integrations in *pchA* and *ptsP* genes were cloned from the wild-type PA01. They were used to confirm gene interruption by the integrating transposon, by comparing restriction fragment length poly-

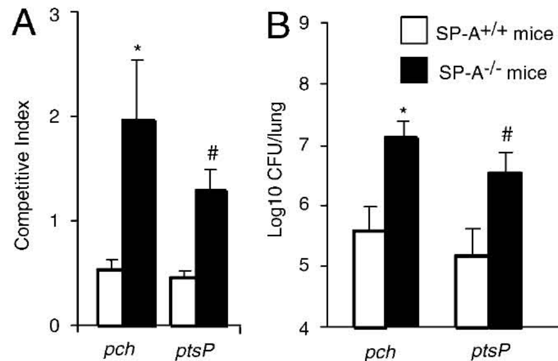

Figure 3. SP-A-Sensitive *P. aeruginosa* STM Mutants Compete Effectively with Parental Wild-Type PA01 in SP-A$^{-/-}$, but Not in SP-A$^{+/+}$ Mice

(A) In vivo competition assays between the wild-type and individual STM mutants after intranasal pulmonary inoculation of 6-wk-old SP-A$^{+/+}$ and SP-A$^{-/-}$ mice. The mean CFU recovered from four mice in each group is shown. The CI is defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria. $*p < 0.013$, $\#p < 0.011$.

(B) Single respiratory tract infection of *pch* and *ptsP* mutants in SP-A$^{+/+}$ and SP-A$^{-/-}$ mice was performed as described for Figure 1. Attenuation is defined as the $\log_{10}$ difference in CFU between wild-type and mutant bacteria recovered from lung tissue 16 h after inoculation. The mean $\pm$ standard error of six mice is shown. $*p < 0.019$, $\#p < 0.045$.

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morphisms (RFLP) between wild-type PA01 and the STM mutants. As shown in Figure 2E, transposon-flanking DNA probes hybridized to DNA fragments with higher molecular weights in the chromosomes of both the *pch* and *ptsP* mutants than their parental strain PA01, indicating transposon insertion.

SP-A-Sensitive STM Mutants Are as Virulent as Parental Wild-Type in SP-A$^{-/-}$ Mice

Apart from confronting pulmonary host defenses, our STM screens require each mutant to compete with 71 other STM mutants within individual library pools. Competitive mixed infection assays have been widely used to assess the fitness of individual mutants versus their parental strains during in vivo infection [16,17]. As shown in Figure 3A, when co-administered with the parental wild-type PA01 by intranasal inoculation into mice, both STM mutants were less competitive than PA01 in SP-A$^{+/+}$ mice. Specifically, mutant strains *pch* and *ptsP* were only 53% and 46% as competitive as PA01, respectively. In contrast, both mutants performed equally well as, if not better than, the wild-type strain in SP-A$^{-/-}$ mice (Figure 3A).

We further tested the mutants, *pch* and *ptsP*, in single infection studies. When infected singly into wild-type mice, the viable bacterial counts of *pch* and *ptsP* mutant bacteria were 32-fold (1.5 log) and 25-fold (1.4 log) higher, respectively, in SP-A$^{-/-}$ mice than in SP-A$^{+/+}$ mice (Figure 3B). Our results indicate that SP-A plays an important role in clearance of PA01 mutants with defective *pch* or *ptsP* gene function.

Preferential Clearance of the STM Mutants from SP-A$^{+/+}$ Lung Is Independent of Macrophages

Previous studies have suggested that SP-A opsonizes *P. aeruginosa* and increases uptake by macrophages [10,18]. To

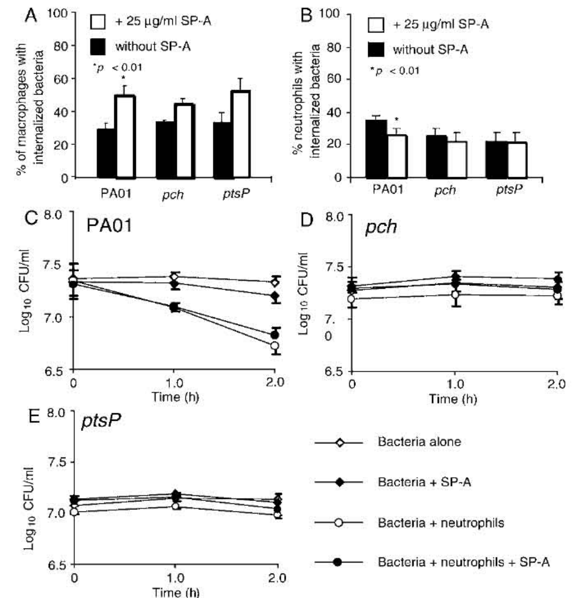

Figure 4. Bacterial Opsonization by SP-A Does Not Result in Differential In Vivo Phagocytosis and Killing of STM Mutants

(A) In vitro phagocytosis assays were performed using live, GFP-expressing PA01 and isogenic *pch and ptsP* bacteria, with alveolar macrophages isolated from SP-A$^{+/+}$ mice. Internalized bacteria were counted using a phase contrast fluorescence microscope. The means of three experiments are shown. Two hundred macrophages were counted for each mouse. $p = 0.0058$ (PA01 vs PA01 + SP-A), $p = 0.075$ (*pch* vs *pch* + SP-A), and $p = 0.109$ (*ptsP* vs *ptsP* + SP-A).

(B) In vitro phagocytosis of PA01, *pch,* and *ptsP* by human neutrophils isolated from healthy volunteers. Phagocytosis experiments were performed as described for (A). $p = 0.037$ (PA01 vs PA01 + SP-A), $p = 0.577$ (*pch* vs *pch* + SP-A), and $p = 0.919$ (*ptsP* vs *ptsP* + SP-A).

(C–E) SP-A did not affect uptake or killing of STM mutants. PA01 (C), but not the STM mutants *pch* (D) and *ptsP* (E) were killed in significant amounts by neutrophils, independent of SP-A. The mean of three separate experiments is shown. $p < 0.001$ when comparing PA01 versus PA01 + neutrophils, and when comparing PA01-SP-A versus PA01-SP-A + neutrophils.

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determine if SP-A-mediated opsonization contributes to the early clearance of STM mutants, we compared the phagocytosis of *pch* and *ptsP* to PA01 in the presence and absence of SP-A. When live, green fluorescence protein (GFP)-expressing bacteria were exposed to mouse alveolar macrophages in the presence of SP-A, the uptake of PA01, its *pch*, or *ptsP* mutant was increased by 1.8, 1.3, and 1.6 fold, respectively, over that which occurred in the absence of SP-A (Figure 4A). However, the SP-A mediated increase in opsonization was only statistically significant in PA01 ($p<0.01$). The magnitude of the increase in the uptake of PA01 in the presence of SP-A was similar to previously published reports [10,18]. Although the addition of SP-A increased the macrophage uptake of STM mutants, enhancement of phagocytosis was slightly lower than that of wild-type PA01. Therefore, it does not appear that SP-A-mediated opsonization is responsible for the preferential clearance of *pch* and *ptsP* mutants from SP-A$^{+/+}$ mice.

Preferential Clearance of the STM Mutants in SP-A$^{+/+}$ Lung Is Independent of Neutrophils

Neutrophils are known to play a major role in protecting the lung against bacterial infection. We examined whether bacterial phagocytosis and killing by neutrophils might have led to preferential clearance of *pch* and *ptsP*. As shown in Figure 4B, the uptake of PA01 and the *pch* and *ptsP* mutants was not increased in the presence of SP-A. In fact, SP-A-opsonized PA01 bacteria were phagocytosed less efficiently by neutrophils than untreated bacteria. Interestingly, only wild-type PA01 bacteria were susceptible to killing by neutrophils, with approximately 73.7% killed in the absence of SP-A, and 57.2% killed in the presence of SP-A (Figure 4C). In contrast, both *pch* and *ptsP* mutants were not susceptible to neutrophil killing (Figure 4D and 4E). These results indicate that neutrophils kill *P. aeruginosa* by using a mechanism independent of SP-A opsonization, and lend support to the notion that neutrophils play a less critical role in the preferential clearance of *pch* and *ptsP* mutants from the SP-A$^{+/+}$ mice than other anti-pseudomonal mechanisms.

The Loss of *pch* and *ptsP* Gene Functions Render *P. aeruginosa* Susceptible to SP-A-Mediated Membrane Permeabilization

Recent studies have indicated that SP-A is capable of directly killing bacteria and fungi by membrane permeabilization and inhibition of macro-molecular synthesis, independent of macrophage-mediated phagocytosis [6,7]. We examined whether direct membrane permeabilization contributed to the clearance of the SP-A-sensitive mutants. Due to the previously described importance of LPS in resistance to SP-A-mediated membrane permeabilization [6,19], we used a LPS mutant *wbpL*, which is deficient in the production of "initial" glycosyltransferase essential for the biosynthesis of both the A-band and B-band O-antigen of LPS [20], as positive control for membrane permeabilization assays. Permeabilization of the *wbpL* mutant was approximately 2.7-fold greater than the wild-type strain PA01 at 60 min (Figure 5A). In contrast, *STMG2A7*, an irrelevant mutant that is virulent in both SP-A$^{+/+}$ and SP-A$^{-/-}$ mice, was found to be as resistant as parental wild-type PA01 to SP-A-mediated membrane permeability (Figure 5B).

The STM mutant *pch*, and a mutant strain, *PA06331,* in which the pyochelin biosynthetic genes encompassing *pchDCBA*, *pchR*, and *pchEFGHI* operons had been removed [14], were permeabilized by SP-A to an extent that was 3.4- and 2.55-fold greater than the wild-type strain PA01, respectively (Figure 5C and 5D) after 60–90 min exposure to SP-A. Similarly, STM mutant *ptsP*, and an in-frame, nonpolar deletion mutant, Δ*ptsP,* were permeabilized by SP-A to an extent that was 3.0- and 3.8-fold greater than the wild-type strain PA01, respectively (Figure 5E and 5F), at 60–90 min post-exposure to SP-A. These data are comparable to the approximately 3-fold increase in membrane permeability that SP-A exposure induces in *E. coli* K12 [6].

SP-A-Mediated Membrane Permeabilization Directly Kills the *pch* and *ptsP* Mutants

We next determined if SP-A mediated permeability kills STM mutants. Live/dead staining, based on exclusion of propidium iodide from the live cells, was performed on bacterial cells following membrane permeability assays.

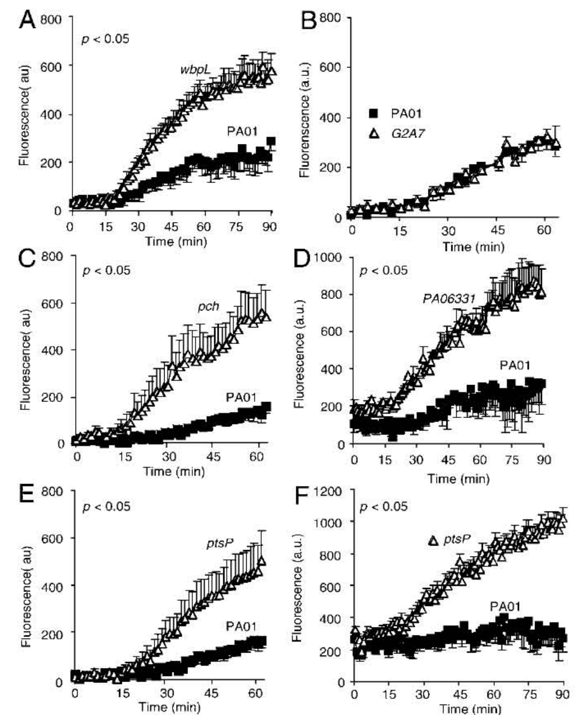

Figure 5. The *pch* and *ptsP* Mutants Are Preferentially Permeabilized and Killed by SP-A

(A) Fluorescence of a cleavage-activated phosphatase substrate ELF97 was examined in the presence or absence of 50 μg hSP-A for a period of 60–90 min. Human SP-A (hSP-A) preferentially permeabilized LPS mutant *wbpL*, but not the parental wild-type PA01. The *wbpL* strain was used as positive control for membrane permeability. The difference in membrane permeabilization between *wbpL* mutant and PA01 was significant from the 28th min onward. $*p < 0.05$.

(B) A mutant strain *STMG2A7* that is virulent in both SP-A$^{+/+}$ and SP-A$^{-/-}$ mice was as resistant to SP-A-mediated membrane permeabilization as the parental wild-type PA01.

(C–D) Membrane permeability of the STM mutant *pch* and *PA06331*, a *P. aeruginosa* strain which is deleted in all of the structural genes required for pyochelin biosynthesis (Table 1.). The difference in membrane permeabilization between the mutants against wild-type PA01 was significant from the 35th and 18th min onward for mutant strains *pch* and *PA06331*, respectively. $*p < 0.05$.

(E–F) Membrane permeability of the STM mutant *ptsP* and the Δ*ptsP*, a *P. aeruginosa* strain with a nonpolar, inframe deletion of the *ptsP* gene. The difference in membrane permeabilization between the mutants against wild-type PA01 was significant from the 29th and 28th min onward, for mutant strains *ptsP* and Δ*ptsP*, respectively. $*p < 0.05$.

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Viable cells are stained green while dead cells are stained red. As shown in Figure 6A, SP-A did not kill wild-type PA01 (green stained). In contrast, mixtures of green- and red-stained cells of *pch* and *ptsP* were visible following treatment with SP-A. Approximately 11% and 9 % of *pch* and *ptsP* cells, respectively, were killed (Figure 6B) within 60 min, in comparison to about 40% of *E. coli* K12 (unpublished data). In addition, in contrast to the robust SP-A-induced aggregation of *E. coli* K12 (Figure 6A), SP-A did not aggregate *P. aeruginosa* PA01, *pch*, or *ptsP*. These results suggest that the loss of intact LPS (i.e. rough LPS), or the inability to synthesize

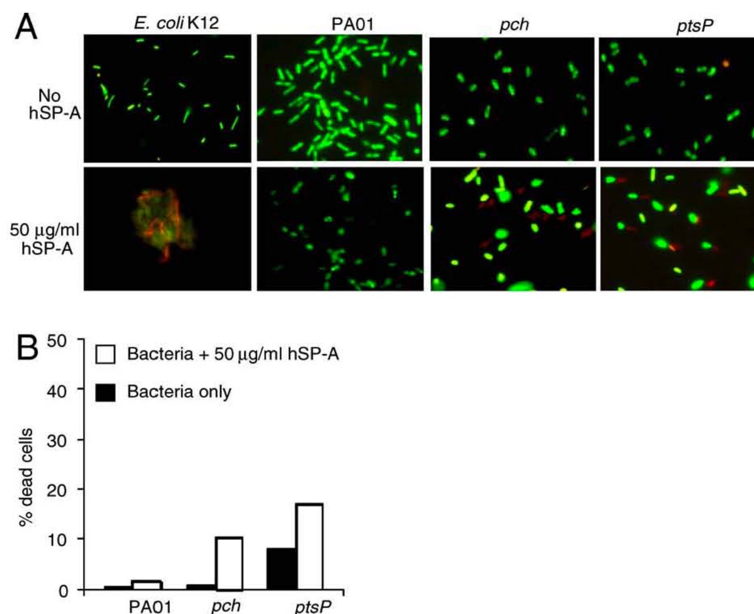

Figure 6. SP-A-Mediated Membrane Permeabilization Directly Kills the *pch* and *ptsP* Mutants

(A) Live/Dead staining was performed on *E. coli* K12, PA01, *pch*, and *ptsP* cells, following 1 h membrane permeabilization with 100 µg/ml SP-A. Green-stained cells are alive whereas red-stained cells are dead.

(B) Enumeration of live or dead bacteria following a 1 h exposure to hSP-A. At least 600–1,000 bacterial cells were counted under a fluorescence microscope. The mean of two experiments is shown.

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salicylate and phosphoenolpyruvate-protein-phosphotransferase, renders the cells vulnerable to membrane permeabilization and killing by SP-A. Furthermore, bacterial aggregation does not play a role in preferential clearance of the *pch* and *ptsP* mutants.

Complementation of the *pch* and *ptsP* Mutants Restores Their Resistance to SP-A-Mediated Membrane Permeabilization

The susceptibility of STM mutants to SP-A mediated membrane permeabilization can be exploited for complementation analyses. As the *pch* mutant is disrupted in a gene required for salicylate biosynthesis, we performed complementation analysis by growing the *pch* mutant bacteria in the presence of 1 mM sodium salicylate. As shown in Figure 7A, the *pch* mutant bacteria grown in LB were susceptible to SP-A-mediated membrane permeabilization. In contrast, the *pch* mutant bacteria grown in LB supplemented with 1 mM sodium salicylate were fully resistant to membrane permeabilization by SP-A. We further examined whether the increased susceptibility to SP-A-mediated membrane permeability was due to the inability *pch* bacteria to synthesize the siderophore pyochelin. The *pch* bacteria cultured in pyochelin-supplemented LB or iron-deficient minimal medium were still membrane permeabilized by SP-A (unpublished data). Collectively, these results suggest that the increased susceptibility to SP-A-mediated membrane permeability in the *pch* bacteria is due to their inability to synthesize salicylate, or both salicylate and pyochelin. However, we cannot completely rule out the possibility that pyochelin may be involved in resistance to SP-A-mediated membrane permeability until we have exhausted all the growth conditions and optimal pyochelin concentrations for the assays.

To rule out the possibility that 1 mM sodium salicylate may inactivate the enzymatic activity of the periplasmic phosphatases that cleaves the impermeant substrate, ELF97, in our permeability assay, we also tested *ptsP* mutant bacteria cultured in the presence or absence of 1 mM sodium salicylate. The *ptsP* mutant bacteria were permeabilized to the same extent, regardless of whether they were grown in the presence or absence of 1 mM sodium salicylate (Figure 7B), suggesting that salicylate did not inactivate phosphatase. These results suggest that the loss of *pch* gene function results in susceptibility of *P. aeruginosa* to clearance by SP-A by rendering the organism susceptible to membrane permeabilization.

We next tested if providing the wild-type *ptsP* gene *in trans* to the *ptsP* STM mutant bacteria could restore the resistance to SP-A-mediated membrane permeabilization. As shown in Figure 7C, the resulting strain, *pUCP-ptsP* was as resistant to SP-A-mediated membrane permeabilization as the wild-type PA01. In contrast, providing an irrelevant *gfp* gene in the *ptsP* bacteria (*ptsP-gfp*) did not restore the resistance of *ptsP* STM mutant to SP-A, nor did it alter the resistance or susceptibility of the parental wild-type PA01 (PA01-*gfp*) to permeabilization by SP-A (Figure 7C). These results suggest that *ptsP* gene function protects *P. aeruginosa* from membrane permeabilization by SP-A.

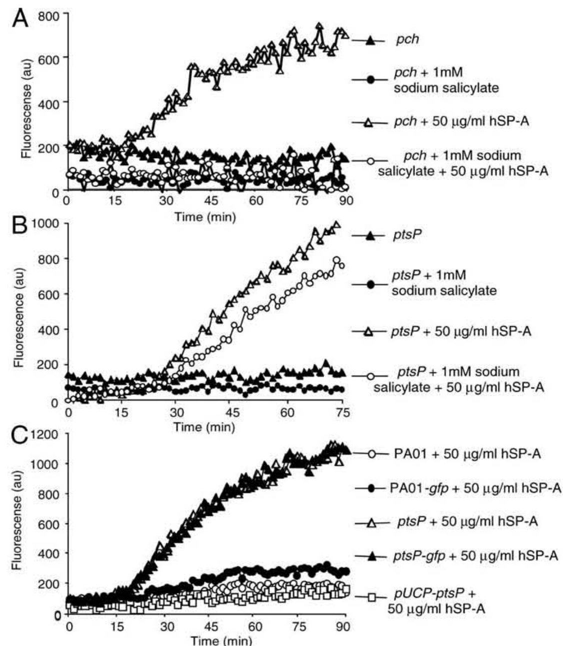

Figure 7. Complementation Studies of SP-A-Sensitive Mutants *pch and ptsP*

(A) Culturing *pch* bacteria in LB supplemented with sodium salicylate restored its resistance to SP-A mediated membrane permeabilization.

(B) In control experiments, we demonstrated that the enzymatic activity of bacterial phosphatases in *ptsP* bacterial cells was not inhibited by 1 mM sodium salicylate.

(C) Provision of the wild-type *ptsP* gene on the pUCP19 *in trans (pUCP-ptsP)* restores the ability of STM mutant *ptsP* to resist SP-A mediated membrane permeabilization. In contrast, *ptsP* mutant expressing GFP gene on the pUCP19 *(pstP-gfp)* is permeabilized to similar levels as *ptsP*.

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LPS Profiles of STM Mutants

Previous studies in *E. coli* [6] and *Bordetella pertussis* [19] have demonstrated that the structures of LPS on the surface of bacterial cells determine resistance to SP-A-mediated aggregation and membrane permeabilization. *P. aeruginosa* LPS mutants are also sensitive to SP-A-mediated membrane permeability, but are not aggregated by the collectin (Figure 5A; unpublished data). To examine if the *pch* and *ptsp* mutants have LPS defects, we compared the LPS profiles after separation by SDS-PAGE and silver staining. The LPS from wild-type PA01 and the *pch* mutant were indistinguishable (unpublished data). However, the two major LPS core bands of the *ptsP* mutant showed a reversal in relative abundance compared with the PA01 (unpublished data). Thus, there may be changes in LPS, at least in terms of the proportion of two different cores made. More detailed molecular analyses will be required to determine if LPS modification occurs within *pch* and *ptsP* mutants.

Discussion

The genome-wide mutagenesis scheme called STM was originally developed to identify novel vaccine and antibiotic targets [9]. STM relies on the ability of the pathogen in question to replicate in vivo as a mixed population, and allows the identification of the virulence genes whose mutant phenotypes cannot be trans-complemented by other virulent strains present in the same inoculum. Hundreds of virulence factors and putative drug targets have been identified by STM in at least 20 different bacterial and fungal pathogens [21]. In all of these screens, a pathogen of interest is mutated by transposon or suicide vector-based insertional strategies. The vectors are each individually tagged with a DNA oligonucleotide to allow mutant identification after selection within the host. Attenuated insertion mutants that are killed by host defense mechanisms such as exposure to SP-A, will fail to amplify from lung homogenates harvested 16 h after intratracheal inoculation (Figure 2A). Comparative STM screening using wild-type and genetically altered mice, as presented herein, represents a novel application of the STM technique. This approach allows the identification of bacterial determinants targeted by specific components of host defense, rather than the entire immune system as a whole. Thus, in contrast to conventional STM screens in wild-type mice, which yield an attenuated mutant recovery rate of 2%–10% [21], our comparative STM screen to identify factors that protect bacteria against a specific host innate immunity protein, SP-A, had a yield of only 0.12%. As STM screens, by definition, only recover mutations in non-essential genes, another reason for detecting few mutants in this screen is that these genes involved in resistance to SP-A may be essential.

In this study, we have employed STM technology to identify bacterial factors that are required for *P. aeruginosa* strain PA01 to confer resistance to SP-A. Both mutants were susceptible to SP-A-mediated membrane permeabilization, confirming that disruption of membrane integrity is one of the mechanisms by which SP-A mediates bacterial clearance. The degree of bacterial membrane permeabilization of the STM mutants by SP-A was comparable to that of *E. coli* K12, which has rough LPS [6]. Based on the results of in vitro assays, macrophages and neutrophils do not seem to play important roles in preferential clearance of the *pch* and *ptsP* mutants. However, we cannot exclude the possibility that phagocytosis contributes to the differential clearance of these two mutants in vivo.

Thus far, we have not recovered STM mutants that are susceptible to SP-A-mediated opsonization, or mutants that are disrupted in the genes required for LPS biosynthesis, which play critical roles in resistance to SP-A-mediated membrane permeability. The lack of recovery of opsonization-sensitive and LPS mutants are not particularly surprising, considering that we screened only approximately 30% of the 5,570 mutants required for 1 × genome coverage (www.pseudomonas.com). Additional screenings are being carried out to catalog other *P. aeruginosa* factors conferring resistance to SP-A.

The mechanisms by which mutations in *pch* and *ptsP* genes result in susceptibility to SP-A-mediated clearance are briefly discussed below. The *pchDCBA* gene encodes enzymes involved in the synthesis of iron-chelating siderophores salicylate and pyochelin [12–14]. In another closely related pathogen, *Burkholderia cepacia,* salicylate is a major siderophore [22]. Salicylate has also been reported to chelate magnesium (Mg^{2+}) and calcium [23]. Thus, it is possible that *P. aeruginosa* requires salicylate and its downstream product

Table 1. Bacterial Strains and Plasmids Used in this Work

Bacterial Strains	Relevant Characteristics	Reference
E. coli		
DH5α	*F-f80 ΔlacZΔM15 endA1 recA1 hsdR17 (r km$^+$k) supe44 thi-1 l-gyr A96 relA1 Δ(lacZYA-argF) U169*	[40]
SM17λpir	*F araD Δ(lac pro) argE(Am) recA56 rif^r nalA*	[41]
P. aeruginosa		
PA01	Wild-type prototrophic laboratory strain	[42]
PA01-gfp	PA01 harboring pUCP19-*gfp*	This study
Pch	PA01 miniTn5Km2*pch*, salicylate and siderophore biosynthesis mutant	This study
PA06331	PA01 harboring deletions in *pchDCBA*, *pchR*, and *pchEFGHI* genes	[14]
ptsP	PA01 miniTn5Km2*ptsP*, phosphoenolpyruvate phosphotransferase mutant	This study
pUCP-ptsP	PA01 miniTn5Km2*ptsP* expressing the wild-type *ptsP* gene on the pUCP19 plasmid	This study
ΔptsP	PA01 harboring non-polar deletion in *ptsP* gene	This study
Plasmids		
pUCP19	polylinker *lacZ*, lac^{iq} selection, *bla*	[44]
pEX18T	polylinker *lacZα*, *bla*, *sacB*	[43]

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pyochelin to acquire cations. Divalent cations, especially Mg^{2+}, are required to structurally stabilize LPS on the outer membrane. The recently resolved structure of SP-A protein indicates that there are two metal binding domains within SP-A, which are most likely occupied by calcium [24]. Thus, SP-A may sequester cations from alveolar lining fluid and result in a growth disadvantage for the salicylate-deficient *pch* mutant. Furthermore, inability to acquire cations may potentially alter the expression, stability, and modification of various transmembrane proteins and glycolipids by enzymes that require cations as cofactors. Thus, we speculate that the *pch* mutation may have contributed to its increased sensitivity to SP-A-mediated membrane permeabilization through direct or indirect effects on LPS integrity and membrane stability.

The *ptsP* gene encodes phosphoenolpyruvate-protein phosphotransferase EI^{Ntr} [15], a transcriptional regulator of RpoN-dependent operons [25]. RpoN is known to regulate the expression of variety of virulence factors in pathogenic bacteria, including the upregulation of the *rfaH* gene, which is required for increased production of the O-antigen of LPS in *Salmonella enterica serovar Typhi* during the stationary phase of growth [25]. The additional O-antigen production during the stationary phase of bacterial growth may be necessary for resistance to SP-A-mediated membrane permeabilization. *PtsP* also coordinates carbon and nitrogen metabolism and is required for virulence in *P. aeruginosa*, by an unknown mechanism [26]. Mutational inactivation of the *Azotobacter vinelandii ptsP* ortholog affects lipidic poly-hydroxybutyrate accumulation within its cytoplasm as a carbon source for long-term starvation survival [27]. Interestingly, poly-hydroxybutyrate is found in the plasma membranes of *E. coli* complexed to calcium polyphosphate, and forms divalent cation-selective channels [28]. Thus, increased sensitivity to SP-A-mediated membrane permeabilization in *ptsP* mutants may be caused by its reduced ability to transport divalent cations necessary to maintain LPS and outer membrane integrity.

Our results suggest that the killing of *P. aeruginosa* PA01 and STM mutants is more robust in vivo than in vitro. One potential explanation for this discrepancy is that our in vitro membrane permeability conditions do not accurately model the environment in the lung. In addition, it is possible that multiple factors contribute to the killing of *P. aeruginosa* including SP-A, SP-D, lactoferrin, β-defensins, and other antimicrobial peptides and proteins [29,30]. We are currently examining whether these peptides act synergistically or cooperatively with SP-A in clearance of *P. aeruginosa*.

Decreased SP-A levels have been found in several respiratory diseases including bacterial pneumonia, adult respiratory distress syndrome [31,32], and cystic fibrosis [33–35]. There is some in vitro evidence to suggest that decreased SP-A and SP-D levels in some of these disease states result from degradation by neutrophil proteases of the host [36,37], or by proteases of bacterial origin [38]. These proteases include bacterial elastases, and neutrophil-derived cathepsin G, elastase, and proteinase-3. The reduced levels of SP-A and SP-D in these lung diseases may contribute to increased susceptibility to infections by variety of microbial pathogens. Degradation of SP-A by *P. aeruginosa* proteases may also explain our failure to develop informative in vitro STM screens (unpublished data).

Antibiotic-resistant *P. aeruginosa* is an emerging clinical problem that can lead to denial for lung transplantation and death [39]. Thus, there is an added urgency to explore the use of novel, non-antibiotic-based, anti-*Pseudomonal* peptides to combat life-threatening infections with this organism. The discovery of factors governing resistance or susceptibility to SP-A may ultimately have therapeutic value.

Materials and Methods

Bacterial strains, media, and growth conditions. Shaking cultures of *E. coli* DH5-α [40], SM17λpir [41], and *P. aeruginosa* strain PA01 [42] were grown in LB broth at 37 °C. When needed, LB was supplemented with 1.5% of bacto agar. Antibiotic selections were performed for *E. coli* or *P. aeruginosa* using the following concentrations: carbenicillin (50 or 300 μg/ml), kanamycin (50 or 250 μg/ml), and tetracycline (5 or 15–30 μg/ml).

Animal husbandry. The C3H/HeN SP-A$^{-/-}$ mice were developed as previously described [6,7]. C3H/HeN control (SP-A$^{+/+}$) mice were purchased from Charles River Laboratory (Boston, Massachusetts, United States). All infections were performed intranasally with age-matched, 6-wk-old mice. Animals were housed in positively ventilated microisolator cages with automatic recirculating water, located in a room with laminar, high-efficiency, particle accumulation-filtered air. The animals received autoclaved food, water, and bedding. Mice used in experimental procedures were handled in accordance with

protocols approved by the Institutional Animal Care and Use Committee at University of Cincinnati College of Medicine.

Comparative screening of the *P. aeruginosa* STM library in SP-A$^{+/+}$ and SP-A$^{-/-}$ mice. The *P. aeruginosa* PA01 STM library and screening methods were previously described [11]. An adapted acute mouse lung pneumonia model of infection was employed, where adult mice rather than the infant mice were used [16]. In earlier experiments, we detected no significant difference in SP-A expression by ELISA assays at 8, 16, and 24 h in SP-A$^{+/+}$ mouse lungs infected with bacteria (1×10^7 cells) from a representative pool of STM library (unpublished data). We chose the 16 h time point to screen the STM library because it gave us the most reproducible PCR amplification of DNA tags from mutants, with $\geq$ 10,000 colony forming units (CFU) still recoverable from the SP-A$^{+/+}$ mice (unpublished data). In contrast, prolonged infection ($\geq$ 36 h) with 1×10^7 bacteria from STM pool frequently killed SP-A$^{-/-}$ mice (unpublished data). Briefly, SP-A$^{+/+}$ and SP-A$^{-/-}$ mice [6,7] were anaesthetized using isofluorane, and were intranasally inoculated with 50 µl (1×10^7 cells) of each individual STM pool. After 16 h, lungs were removed from sacrificed mice, and homogenized tissues were plated on LB agar plates. At least 10,000 bacterial colonies recovered after in vivo selection were used for multiplex PCR as described previously [11]. Twenty sets of 72 mutants (total = 1,440) were pooled and screened. An additional 240 mutants previously shown to be attenuated in a chronic model of rat lung infection [11] were also screened. The mutants that were differentially cleared by SP-A during the in vivo passaging were subsequently retested by single PCR in bacterial cells recovered from three separate mice.

In vivo competitive and single infection assays. For competition assays, mouse lungs were inoculated intranasally with bacterial cells (1×10^7 in 50 µl) composed of a 1:1 ratio of wild-type PA01 and its isogenic STM mutants. Infected lungs were recovered 16 h after infection for bacterial load determination. The competitive index (CI) is defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria [16,17]. Thus, if a mutant strain is less competitive than the parental strain from which it was derived, a CI value of < 1 will be achieved, indicating that the mutant is attenuated. Single organism inoculations with individual bacterial strains were performed by the intranasal route in SP-A$^{+/+}$ and SP-A$^{-/-}$ mice (group of six). Attenuation was defined as the $\log_{10}$ difference in CFU between wild-type and mutant bacteria recovered from lung tissue 16 h after inoculation.

Mutant cloning and bioinformatics analysis. Cloning of putative STM mutants were performed as previously described [11]. The DNA sequences were analyzed with MacVector or DNA Star software. Sequence data was compared with the database available for *P. aeruginosa* at http://www.pseudomonas.com.

Non-polar deletion and complementation of the *ptsP* mutant. Non-polar, in-frame deletion of *ptsP* was generated by PCR: 0.802 and 1.155-kb 5′ and 3′ segments were amplified from target PA01 genomic DNA, and each amplicon, which included an engineered restriction site, was ligated into pEX18Ap [43] to produce replacement plasmids. Inframe deletion mutants were generated in PA01 via homologous recombination by sucrose resistance selection, and confirmed by hybridization and PCR analysis (unpublished data). To complement the *ptsP* mutation, a 4.5-kb DNA fragment containing both the promoter and the *ygdP-ptsP-PA0338* operon (Figure 2C) was PCR-amplified from the wild-type parental strain PA01 using the Expand Long Template PCR System (Roche Diagnostic, Basel, Switzerland), and cloned into *E. coli-P. aeruginosa* shuttle plasmid pUCP19 [44] to obtain pUCP19-*ptsP*. The cloned fragment was sequenced to rule out any mutation that was introduced during the amplification. The pUCP19-*ptsP* was introduced into *P. aeruginosa* STM mutant strain *ptsP* by heat shock. Carbenicillin-resistant transformants were selected, and verified by Southern hybridization and PCR methods (unpublished data). Complementation was assessed by resistance to SP-A-mediated membrane permeabilization.

Complementation of the *pch* STM mutant. Complementation of the *pch* mutant was performed by culturing the bacteria into stationary phase in LB supplemented with 1 mM sodium salicylate.

Pyochelin extraction and detection. Pyochelin production in *P. aeruginosa* was analyzed using the method previously described [45]. M63 minimal medium (5 ml) was inoculated with fresh colonies of individual *P. aeruginosa* strains and incubated at 37 °C for 24 h with shaking. A fresh 30 ml of M63 supplemented with 0.5% w/v casamino acids was then inoculated with 0.3 ml of starter culture, and incubated for 36 h at 37 °C with shaking. The culture was then centrifuged at 13,000 rpm for 10 min in 1.5 ml aliquots in a microcentrifuge. The supernatants were removed into a 50 ml polypropylene screw-capped centrifuge tube, pooled, adjusted to (pH 2.0) with 1M HCl, using universal indicator paper to estimate pH, and filter sterilized through a 0.22 µM membrane. Pyochelin was extracted by the addition of 0.4 volumes of ethyl acetate, followed by vigorous vortexing. The two phases were separated by centrifugation at 2,000 rpm for 5 min in a bench top centrifuge, and the upper, organic phase was removed to a separate tube. Pyochelin extract was concentrated by rotary evaporation at 40 °C in 100 ml round bottomed flasks, and the residue was dissolved in 300 µl of methanol. Pyochelin extracts (10 µl) was separated by TLC (silica gel 60, plastic backed, from Sigma, St. Louis, Missouri, United States), using acetone:methanol:0.2M acetic acid (5:2:1) as a development solvent. The chromatography tank was left to stand for several minutes with its lid on to allow the air inside to become saturated with solvent vapor. Pyochelin, which is fluorescent, naturally occurs as two stereoisomers. When visualized under a UV transilluminator, it is revealed as two green fluorescent bands. Pyochelin standard was generously provided by Dr. J. M. Meyer (Université Louis Pasteur).

Purification of human SP-A. Human SP-A (hSP-A) was purified from lung washings of patients with the lung disease alveolar proteinosis, as previously described [6]. SP-A preparations were resuspended in 5 mM Tris, 150 mM NaCl. SP-A preparations were determined to contain 140–190 pg of LPS/µg of SP-A by the Limulus Amebocyte Lysate (BioWhittaker, Rockland, Maryland, United States).

Assays of *P. aeruginosa* permeability. The effect of the SP-A on *P. aeruginosa* cell wall integrity was assessed by determining permeability to a phosphatase activity substrate, Enzyme-Labeled Fluorescence 97 (ELF-97, Molecular Probes, Eugene, Oregon, United States) as described [7]. SP-A was incubated with 1×10^8 bacterial cells/ml in 100 µl of 5 mM Tris, 150 mM NaCl for 15 min at 37 °C, and 100 µM ELF97 phosphatase substrate was added. Fluorescence was measured at excitation and emission wavelengths of 355 and 535 nm, respectively, for a period of 60–90 min.

In vitro macrophage and neutrophil phagocytosis assays. Freshly lavaged mouse macrophages ($\sim 5 \times 10^5$ from 3 SPA$^{+/+}$ mice) were cultured on chamber plastic culture slides, coated with 5% poly-D-lysine with 200 µl RPMI (Dulbecco's, containing 2.5 mg/L gentamycin and 0.1% BSA) and used immediately. Macrophages were allowed to adhere for 2 h at 37 °C in 5% CO_2. Live GFP-expressing PA01 or isogenic STM mutants in RPMI were opsonized with or without 25 µg/ml human SP-A for 1 h at 37 °C with rotation. The medium was removed from each well and replaced with 250 µl of opsonized PA01 or STM mutants, and incubated for 1 h at 37 °C in 5% CO_2 at a ratio of 100 bacteria to 1 macrophage. Chamber slides were washed three times with PBS containing 1 mM $CaCl_2$. Extracellular bacteria were quenched with crystal violet (0.8 mg/ml). Following two additional washes, cells were fixed with 1% paraformaldehyde in PBS plus 1 mM $CaCl_2$ for 10 min, and stained with Evans blue for 2 min. The percentage of macrophages with engulfed bacteria was quantified under a phase contrast fluorescence microscope. At least 200 macrophages were counted. Human neutrophils were purified and cultured as previously described [46]. Neutrophil phagocytosis was performed as described for macrophages.

LIVE/DEAD bacterial staining. The viability of SP-A exposed bacteria was determined by LIVE/DEAD Baclight^{TM} Bacterial Viability Kit (Molecular Probes). Briefly, stationary phase *E. coli* or *P. aeruginosa* bacteria were washed three times with 5 mM Tris, 150 mM NaCl, and adjusted to an of $OD_{600} = 1.0$. A 100-µl aliquot of the cells was added to culture tubes containing 100 µl of SP-A (100 µg/ml) or 100 µl of 5 mM Tris, 150 mM NaCl, as a control. The mixtures were incubated at 37 °C, 300 rpm for 1 h, and stained according to the instructions provided by the supplier. The viability of the cells was checked under a fluorescence microscope. At least 1, 000 cells were counted.

LPS preparations. LPS was isolated from *P. aeruginosa* by acetone/phenol extraction [47]. LPS samples were size-fractionated on 4%–12% Bis-Tris PAGE gels (NuPAGE Novex Gels; Invitrogen, San Diego, California, United States) and stained with silver.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test and one-way analyses of variance (ANOVA). A significant difference was considered to be $p < 0.05$.

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Competing interests. The authors have declared that no competing interests exist.

Author contributions. SZ, YC, FXM, and GWL conceived and designed the experiments. SZ, YC, FS, and GWL performed the experiments. SZ, YC, FS, FXM, and GWL analyzed the data. EP, FS, RCL, FXM, and GWL contributed reagents/materials/analysis tools. GWL wrote the paper.

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Appendice 3

Symposium du CREFSIP, Mai 2003

CRIBLAGE MOLÉCULAIRE ET IDENTIFICATION DE NOUVELLES CIBLES ANTIBACTÉRIENNES THÉRAPEUTIQUES *IN VIVO* CHEZ *PSEUDOMONAS AERUGINOSA*.

Potvin E, Sanschagrin F, Lehoux D, Levesque RC.
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Pseudomonas aeruginosa est l'agent causal principal de nombreuses infections pulmonaires chroniques mortelles chez les personnes atteintes de la fibrose kystique. Le séquençage du génome (6,3Mb) a permis de mettre en évidence l'organisation complexe d'un chromosome hautement régulé. L'objectif de nos recherches est d'identifier des gènes essentiels *in vivo* suite à la construction d'une banque de 9600 mutants transpositionnels en utilisant une technique de mutagenèse par étiquette (STM, Signature Tagged Mutagenesis). La banque a été criblée dans un modèle d'infection pulmonaire chronique chez le rat et 243 mutants STM ont été démontrés essentiels à l'infection. Des analyses de croissance ainsi que des changements phénotypiques en regard aux facteurs de virulence connus ont été faits sur une sélection de ceux-ci. 9 gènes comportant des homologies avec des protéines connues ou inconnues ont été choisis dans le but d'être caractérisée en profondeur. Soit, un transporteur ABC, une protéine de type IcmF, un récepteur dépendant TonB, une protéine du phage ctx, une NADH déshydrogénase probable, trois protéines conservées hypothétiques, et une protéine de biosynthèse des glucides périplasmiques ont été étudiés en détails. Des données préliminaires ont permis d'associer le récepteur TonB dépendant à un défaut dans la formation du biofilm bactérien et d'autres comme PilY1 et la protéine de type IcmF à une défectuosité de la motilité due aux pilis. De plus, une des protéines hypothétiques semblent contrôler l'expression de protéases extracellulaires reconnues comme étant un facteur tournant à l'établissement d'une infection. La technique de mutagenèse utilisée est appuyée par une sélection *in vivo* de gènes connus en pathogénie de *Pseudomonas aeruginosa*, de plus, les gènes inconnus choisis semblent démontrer une importance capitale dans l'établissement d'une infection chronique.

Appendice 4

53rd Annual Meeting of the Canadian Society of Microbiologists, Mai 2003

High throughput screening of signature-tagged mutagenesis Pseudomonas aeruginosa mutants in a rat model of chronic lung infection.

Eric Potvin, Dario Lehoux, François Sanschagrin and Roger C. Levesque

Université Laval, Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines (CREFSIP), Ste-Foy, Québec.

Pseudomonas aeruginosa is a multi-drug resistant pathogen known to cause chronic lung infection in cystic fibrosis patients. The sequencing of the complete 6,3 Mb genome of strain PAO1 emphasized complex genomic gene expression and environmental sensing by this organism. We identified and characterized essential in-vivo genes, using a PCR-based STM screening of a 9600 *Pseudomonas aeruginosa* mini-Tn5 library of mutants. The STM mutants constructed were screened in vivo; using a chronic lung infection rat model. After 7 days of infection, we isolated 214 STM mutants in vivo; cloning and sequencing of transposon inserted genes permitted to identify 16 classes of genes as defined in various bacterial genomics projects. We decided to focus efforts on 10 genes selected according to their role as essential for bacterial maintenance in vivo and by identity with defined and unknown virulence factors, PA0073 (ABC transporter), PA0077 (Icmf like protein), PA0151 (TonB dependent receptor), PA0620 (ctx tail fiber protein), PA1054 (probable NADH deshydrogenase), PA2895, PA2972, PA4488, PA4491 (conserved hypothetical proteins). We tested each mutant selected for a particular phenotype to assess pleiotropic effects related to known virulence factors such as protease, lipase, lecithinase, elastase, alginate, fluorochrome, hemolysin; biofilm formation; flagellum and twitching motility. These mutants were further characterized using phenotypic tests to associate a potential mutation with a specific phenotype. In depth molecular and biochemical characterization of these genes and their products will give a better understanding of *Pseudomonas aeruginoa* in vivo pathogenicity.

Appendice 5

Journée de la Recherche, Faculté de Médecine, Juin 2003

GÉNOMIQUE BACTÉRIENNE ET INTERACTIONS MOLÉCULAIRES HÔTE-PATHOGÈNE DE *PSEUDOMONAS AERUGINOSA* DANS UN MODÈLE D'INFECTION PULMONAIRE CHRONIQUE CHEZ LE RAT.

Potvin Éric, Sanschagrin François, Lehoux Dario, Levesque Roger C.
Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines (CREFSIP).

Pseudomonas aeruginosa est l'agent causal principal de nombreuses infections pulmonaires chroniques mortelles chez les personnes atteintes de la fibrose kystique. Le séquençage du génome (6,3Mb) a permis de mettre en évidence l'organisation complexe d'un chromosome hautement régulé. **Objectifs :** Nous avons tenté d'identifier des gènes essentiels *in vivo* suite à la construction d'une banque de 9600 mutants mini-Tn5 en utilisant une technique de mutagenèse par étiquette (STM, Signature Tagged Mutagenesis). **Méthodes :** La banque a été criblée dans un modèle d'infection pulmonaire chronique chez le rat et 214 mutants STM ont été démontrés essentiels à l'infection. Des analyses de croissance ainsi que des changements phénotypiques en regard aux facteurs de virulence connus ont été faits. **Résultats :** Nous avons choisi 10 gènes comportant des homologies avec des protéines connues ou inconnues. Un transporteur ABC, une protéine de type IcmF, un récepteur dépendant TonB, une protéine du phage ctx, une NADH déshydrogénase probable, trois protéines conservées hypothétiques, et une protéine de biosynthèse des glucides périplasmiques ont été étudiés en détails. Des données préliminaires ont permis d'associer le récepteur TonB dépendant à un défaut dans la formation du biofilm bactérien et d'autres comme PilY1 et la protéine de type IcmF à une défectuosité de la motilité. **Conclusion :** La technique de mutagenèse utilisée est appuyée par une sélection *in vivo* de gènes connus en pathogénie de *Pseudomonas aeruginosa*, de plus, les gènes inconnus choisis semble démontrer une importance capitale dans l'établissement d'une infection chronique.

Appendice 6

9th International Pseudomonas Meeting, Septembre 2003

In vivo functional genomics of *Pseudomonas aeruginosa* for high throughput screening of new virulence factors and antibacterial targets.

Eric Potvin[1], Dario Lehoux[1], Irena Kukavica-Ibrulj[1], Karine Richard[1], François Sanschagrin[1], Gee W. Lau[2], and Roger C. Levesque[1]*

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Pseudomonas aeruginosa is a model for studying opportunistic pathogens that are highly resistant to most classes of antibiotics and causing chronic pulmonary infections. We have developed and adapted a multiplex PCR-based signature-tagged mutagenesis for high throughput screening of a collection of 7968 *P. aeruginosa* mutants in a rat model of chronic respiratory infection. After three rounds of screening, a total of 214 mutants representing transposition events into 148 open reading frames, were shown to be attenuated in lung infection and were retained for further analysis. As proof of concept supporting this technology, we identified 11 insertions in typical virulence genes such as those coding for pili implicated in motility, attachment, and swarming, alginate synthesis and its expression, a mucus transcription regulator, extracellular enzymes such as alkaline protease, esterase and amino peptidase, a rhamnosyl surfactant transferase and a LPS glycosyl transferase. Detailed analysis of the 148 STM mutants, including 7 auxotrophs, revealed insertions in 21 of the 26 known genes classes used to characterize sequenced bacterial genomes. We noted that at least 46% of STM mutants identified had insertions in hypothetical or proteins of unknown function and that approximately 40% of all STM mutants had insertions in surface proteins including the outer membrane, the periplasm and the inner membrane. Interestingly, 11 STM mutants attenuated for lung infection were also identified in microarray and transcriptome for quorum sensing and mucus production. The remaining 130 mutants were systematically analyzed for their capability to fully express

known virulence factors. In addition, testing the ability of these mutants to infect alternative model host *Drosophila melanogaster* revealed 36 STM mutants defective in protease, twitching motility, swimming and swarming. Finally, we identified many genes whose activity in respiratory infection was not fully appreciated.

Appendice 7

Symposium du CREFSIP, Mai 2004

IDENTIFICATION D'UNE NOUVELLE CIBLE ANTI-BACTÉRIENNE CONTRÔLANT LA PRODUCTION D'EXOPROTÉASES CHEZ UN MUTANT DE PSEUDOMONAS AERUGINOSA ATTÉNUÉ IN-VIVO.

Potvin E, Sanschagrin F, Levesque R.C.
Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines (CREFSIP), Université Laval.

Pseudomonas aeruginosa est l'agent causal principal d'infections pulmonaires chroniques mortelles chez les personnes atteintes de la fibrose kystique. L'étude du génome de la bactérie par mutagénèse à étiquette a permis de cibler 243 gènes essentiels à l'établissement d'une infection chronique. Nous avons identifié un opéron (PA2895-PA2896) essentiel à l'établissement d'une infection pulmonaire chronique chez le rat par la méthode de la STM (Signature-Tagged Mutagenesis). Des analyses bioinformatiques détaillées ainsi que des tests phénotypiques ont confirmé que l'opéron contrôle la production de deux protéases majeures de *P. aeruginosa*, l'elastase et la staphylolysine. Des analyses moléculaires de types Western et Northern sont en cours dans le but d'identifier si le contrôle s'effectue au niveau transcriptionnel ou post-traductionnel. De plus la surproduction des protéines facilitera leur caractérisation structure-fonction. PA2896 est fortement homologue à un facteur transcriptionnel sigma 70 ECF reconnu comme contrôlant l'expression de gènes dont les fonctions sont extra cytoplasmiques. PA2895 est le gène en aval de PA2896 et ne possède quant à lui aucun homologue et donc aucune fonction prédite mise à part un domaine transmembranaire. Des mutants insertionnels de chacun des gènes ont été construits et testés pour leur production d'elastase. Le gène PA2895 a été démontré comme essentiel à la production efficace des enzymes protéolytiques de la bactérie lorsqu'inactivé et complémenté en trans par une copie du gène sauvage. L'elastase de *Pseudomonas aeruginosa* est un des facteurs de virulence majeurs de la bactérie; ainsi une meilleure compréhension de l'établissement de l'infection pulmonaire chronique via la sécrétion des protéases ouvrira la porte à une nouvelle approche thérapeutique.

Appendice 8

Journée de la Recherche, Faculté de Médecine, Juin 2004

RÉGULATION DE L'ELASTASE ET DE LA STAPHYLOLYSINE DE *PSEUDOMONAS AERUGINOSA* CHEZ UN MUTANT ATTÉNUÉ IN VIVO DANS UN MODÈLE D'INFECTION PULMONAIRE CHRONIQUE CHEZ LE RAT COMME NOUVELLE CIBLE ANTI-BACTÉRIENNE.

Potvin Eric, Sanschagrin François, Levesque Roger C.
Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines (CREFSIP).

OBJECTIF: *Pseudomonas aeruginosa* est l'agent causal principal d'infections pulmonaires chroniques mortelles chez les personnes atteintes de la fibrose kystique. L'étude du génome de la bactérie par mutagénèse à étiquette a permis de cibler 243 gènes essentiels à l'établissement d'une infection chronique. **MÉTHODES:** Nous avons identifié un opéron (PA2895-PA2896) essentiel à l'établissement d'une infection pulmonaire chronique chez le rat par la méthode de la STM (Signature-Tagged Mutagenesis). Des analyses bioinformatiques détaillées ainsi que des tests phénotypiques ont confirmé que l'opéron contrôle la production de deux protéases majeures de *P. aeruginosa*, l'elastase et la staphylolysine. Des analyses moléculaires de types Western et Northern sont en cours dans le but d'identifier si le contrôle s'effectue au niveau transcriptionnel ou post-traductionnel. De plus la surproduction des protéines facilitera leur caractérisation structure-fonction. **RÉSULTATS :** PA2896 est fortement homologue à un facteur transcriptionnel sigma 70 ECF reconnu comme contrôlant l'expression de gènes dont les fonctions sont extra cytoplasmiques. PA2895 est le gène en aval de PA2896 et ne possède quant à lui aucun homologue et donc aucune fonction prédite mise à part un domaine transmembranaire. Des mutants insertionnels de chacun des gènes ont été construits et testés pour leur production d'elastase. Le gène PA2895 a été démontré comme essentiel à la production efficace des enzymes protéolytiques de la bactérie lorsqu'inactivé et complémenté en trans par une copie du gène sauvage. **CONCLUSION :** L'elastase de *Pseudomonas aeruginosa* est un des facteurs de virulence majeurs de la bactérie ; ainsi une meilleure compréhension de l'établissement de l'infection pulmonaire chronique via la sécrétion des protéases ouvrira la porte à une nouvelle approche thérapeutique.

Appendice 9

105th Annual meeting of the American Society for Microbiology, Juin 2005

Functional Genomics of an Essential *In Vivo* Sigma Factor Regulating Extracytoplasmic Proteases from *Pseudomonas aeruginosa*.

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Background: *Pseudomonas aeruginosa* (PA) is the major cause of lethal chronic lung infection in Cystic Fibrosis and immunocompromised patients. The recent completion and exhaustive analysis of the 6.3Mb genome sequence corroborate a complex genomic organization and expression of highly regulated genes for this opportunistic pathogen. **Methods:** A detailed functional genomic study by high-throughput screening of the complete genome of PA using Signature-Tagged Mutagenesis (STM) containing 7968 STM mutants identified 148 ORFs essential for establishment of an infection in the rat chronic lung infection model. We selected STM2895 as a prototype STM mutant defective in *in vivo* maintenance which was found to have deficiencies in protease production. Characterization of the two gene PA2895-PA2896 operon was performed using bioinformatics analyses, western blotting, biochemical assays, DNA microarrays, allelic exchange and *in vivo* competitive index measurements. **Results:** Detailed bioinformatics analyses indicated that the PA2896 is 57% homologous to an ECF sigma-70 factor and PA2895 a putative anti-sigma. Western blots using anti-LasA and anti-LasB antibodies and protease biochemical assays showed the accumulation of non-functional but correctly processed LasA protease and the low abundance of non-functional LasB elastase. DNA microarrays were used to analyse the STM2895 mutant; 67 ORFs were shown to be differentially expressed, 47 upregulated among which PA0323, PA4495, PA1494 and PA2896. In the 20 downregulated gene pool, PA4683, PA3568 and PA2719 are the most significant. Competitive index with wild-type strain PAO1, PAO1Δ2895::Gm^r confirmed attenuation *in vivo* using the rat lung model. **Conclusion:** Characterization of novel

sigma/anti-sigma system PA2895-PA2896 redefines the expression of PA exoproteases *in vivo*.

Appendice 10

10th Pseudomonas International Meeting, Août 2005

Functional genomics of an essential in vivo sigma factor regulating extracytoplasmic proteases from *Pseudomonas aeruginosa*.

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By secreting a large arsenal of virulence factors, *P. aeruginosa* causes lethal respiratory failure in CF patients. To understand host/bacterium interactions, we constructed a library of *P. aeruginosa* (PAO1) mutants using PCR-Based Signature-Tagged Mutagenesis. The mutant collection was screened in the rat model of chronic lung infection and identified 148 essential *in vivo* genes. A mutant of PA2895 gene (STM2895), frequently recovered by the STM screening, was primarily identified by its exoproteases defect. Biochemical investigations using elastin congo-red, staphylolytic assay and poly-L-lysine degradation assays revealed that STM2895 was affected in LasA, LasB and lysine proteases. Immunoblots of STM2895 showed that LasB was present and correctly refolded and inactive whereas LasA accumulates in a processed form. A deletion Gm^R mutant of PA2896 was constructed and used with STM2895 in microarray experiments. Compared to wild-type PAO1, 128 genes (64 up and 64 down) for STM2895 and 138 genes (59 up and 79 down) for ΔPA2896::Gm were differentially regulated at ≥ 5 fold. Results from both STM2895 and ΔPA2896::Gm, where comparable in 76% of cases for upregulation and 59% for downregulation. Of significance is the number of operons or gene clusters involved in extracellular functions and genes associated to virulence (type III secretion pathway, exoenzymes, secondary metabolites and siderophores production). For repressed genes, 4 sigma-70 and PvdS (-24 fold) were highlighted as well as several genes identified with microarray data monitoring iron metabolism. In the STM2895 background, PA2896 was upregulated more than 9 fold confirming the negative regulation hypothesis and

suggesting a feedback loop regulation. Competitive index (CI) confirmed the attenuation *in vivo* of STM2895 and a deleted mutant ΔPA2895::Gm had CI values of 0.074 and 0.116, respectively. We conclude that PA2895 is a negative regulator of PA2896 ECF sigma-70 factor and plays a major role in virulence.