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Outcome and Prevention of *Pseudomonas aeruginosa*-*Staphylococcus aureus* Interactions During Pulmonary Infections in Cystic Fibrosis

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1. Introduction

Several microorganisms take advantages of the most common single gene disorder afflicting Caucasians and colonize the airways of cystic fibrosis (CF) patients. Although CF is a multi-system disorder, the associated mortality is mostly due to respiratory problems subsequent to chronic bacterial infections. CF is the result of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) which is a cAMP-regulated chloride channel and a regulator of the activity of other channels. Consequently, there is a significant dehydration of the airway mucus when CFTR is dysfunctional. However, the exact reason why CF predisposes the lungs to microbial infections is not completely understood. It is thought that the obstruction of the airways by mucus and the proinflammatory status associated with this disease may be part of the explanation (Lyczak *et al.*, 2002; Riordan, 2008).

The recalcitrance of CF pathogens to antibiotic therapies is a major problem and is responsible for most of the morbidity and the mortality associated with CF (Chmiel & Davis, 2003; Lyczak *et al.*, 2002). The ever-growing and overwhelming problems caused by antibiotic-resistant bacteria in human medicine have not spared CF patients. Antibiotic-resistant bacteria are frequently recovered from CF samples (George *et al.*, 2009; Parkins & Elborn, 2010). The evolution of drug resistance has been accelerated by the extensive use of an antibiotic arsenal which has become limited due to insufficient innovation in the development of antimicrobials (Shah, 2005; Talbot *et al.*, 2006). Indeed, the development of new classes of antibiotics has been almost abandoned for the last four decades and antibiotics that were introduced during this period usually consisted of new-generation molecules derived from existing antibiotics (Wenzel *et al.*, 2005). Furthermore, long-term infections of the CF airways not only allow pathogens to adapt and circumvent the host immune system, but also allow them time to adapt to antibiotic therapies (Goerke & Wolz, 2010; Hogardt & Heesemann, 2010). Several mechanisms that decrease the susceptibility to antimicrobials are known to be at play in the CF airways. Conventional resistance mechanisms include the upregulation of bacterial efflux pumps and mutations of antibiotic target molecules (Høiby *et al.*, 2010). In addition, the formation of persister cells (Mulcahy *et al.*, 2010) and of small-colony variants (Goerke & Wolz, 2010; Proctor *et al.*, 2006; Schneider *et al.*, 2008) as well as bacterial growth in biofilms (Høiby *et al.*, 2010; Mitchell *et al.*, 2010a,

2010b; Wagner & Iglewski, 2008) are mechanisms that are well known for their involvement in difficult-to-treat infections (Galli *et al.*, 2007; Stewart, 2002).

The formation of bacterial biofilms may explain, at least in part, the failure of many antimicrobial therapies. Biofilm-growing bacteria are highly persistent in CF because they appear to be physically protected from the host immune system and are inherently resistant to antimicrobials (Costerton *et al.*, 1999; Davies & Bilton, 2009; Høiby *et al.*, 2010; Stewart, 2002). These bacteria are thought to be as much as 1000 times more resistant to antimicrobials than their planktonic counterparts (George *et al.*, 2009). Biofilms also represent complex integrated polymicrobial communities that are strongly influenced by cell-to-cell intraspecies and interspecies communications (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Stoodley *et al.*, 2002). The different species of microbes found in polymicrobial infections such as in CF most probably respond to each other's chemical signals in order to survive and this is likely to influence the course of any particular infection (Brogden *et al.*, 2005; Ryan & Dow, 2008).

In the surge to develop new therapies against CF pathogens, multiple aspects should be considered. Indeed, several diverse phenotypic adaptations confer a selective advantage in the host environment or toward antibiotic therapies. Also to consider are the various bacterial signals used for cell-to-cell communication that are involved in the establishment and the development of an infection. These mechanisms enable pathogens from polymicrobial communities to adjust their behavior in response to other neighboring bacteria and they deserve particular attention. This chapter aims to describe some of the mechanisms used by *Pseudomonas aeruginosa* and *Staphylococcus aureus* for their mutual coexistence and persistence in the host environment, and to emphasize those that are potential targets for the development of anti-pathogenesis therapies.

2. Microbiology of CF

Colonization of the CF airways by bacteria usually occurs in infancy and results in the establishment of chronic infections which eventually lead to respiratory failure and death (Harrison, 2007; Lyczak *et al.*, 2002). Recent investigations have shown that the CF airways are colonized by complex polymicrobial communities constituted by numerous bacterial species and not only by the relatively few predominant species originally described that include *P. aeruginosa*, *S. aureus*, *Haemophilus influenzae*, bacteria from the *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia* (Sibley & Surette, 2011). Notwithstanding this polymicrobial mixture, *P. aeruginosa* and *S. aureus* are still among the most prevalent and dominant bacterial species encountered in CF (Canadian Cystic Fibrosis Foundation, 2009; Cystic Fibrosis Foundation, 2009; European Cystic Fibrosis Society, 2007). It is also important to note that the prevalence of the different pathogens varies as a function of the age of patients, with *S. aureus* and *H. influenzae* being more frequent in early childhood and *P. aeruginosa* being more important as patients become older. Several other microorganisms such as *Mycobacterium* spp., pathogenic viruses, fungal pathogens (*e.g.* *Aspergillus fumigatus*) and yeasts (*e.g.* *Candida albicans*) have also been recovered from the CF airways and may also contribute to disease (Harrison, 2007; Lyczak *et al.*, 2002; Moskowitz *et al.*, 2005). It should be kept in mind that the chronically infected CF airways represent a complex and diverse ecosystem and that the precise contribution of the different microbes to the morbidity of the disease remains undetermined, even for the less frequently encountered microorganisms (Harrison, 2007).

2.1 Pathogenesis of *P. aeruginosa* in CF

P. aeruginosa is a Gram-negative bacterium that has the ability to survive in several different natural environments. However, it is better known as an opportunistic antibiotic-resistant human pathogen often encountered in hospital settings (Bodey *et al.*, 1983). This bacterium is also known as the major cause of lung function decline and mortality in CF (Lyczak *et al.*, 2002). *P. aeruginosa* establishes infections using several virulence factors. The production of these virulence factors varies as a function of the cell density of the bacterial population and is controlled by cell-to-cell chemical communication, *i.e.* quorum-sensing or QS (R. S. Smith & Iglewski, 2003). QS systems are used by many bacteria to promote collective behaviors and depend on the action of diffusible signal molecules. QS systems are typically composed of a signal synthase that produces the signal molecule and a signal receptor that modulates the expression of target genes subsequent to the binding of the signal molecule.

QS in *P. aeruginosa* is controlled by the *las* and *rhl* *N*-acylhomoserine lactone (AHL) regulatory circuits and by the 2-alkyl-4-quinolone (AQ) system (Dubern & Diggle, 2008). In the *las* and *rhl* system, the *lasI* and *rhlI* gene products direct the synthesis of the homoserine lactones (HSL) 3-oxo-C12-HSL and C4-HSL, which interact with the transcription regulators LasR and RhlR, respectively, and activate target promoters. The *las* and *rhl* systems are hierarchically connected and interact together to regulate the production of several virulence determinants such as pyocyanin biosynthesis and biofilm formation (Dubern & Diggle, 2008; R. S. Smith & Iglewski, 2003). The AQ system is also interconnected with *las* and *rhl* and leads to the production of 2-heptyl-4-quinolone (HHQ) and the *Pseudomonas* quinolone signal (PQS). Both HHQ and PQS also have a role in cell-to-cell communication. However, PQS has a number of other biologically important functions (Dubern & Diggle, 2008). *P. aeruginosa* produces several other quinolone compounds, some of which, such as 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), have antibiotic activity (Leisinger & Margraff, 1979; Lépine *et al.*, 2004; Machan *et al.*, 1992). It should also be kept in mind that the control of virulence factors by these systems is growth and environment dependent. This complex mixture of factors allows *P. aeruginosa* to modulate its behavior only when necessary (Williams & Camara, 2009). Interestingly, *P. aeruginosa* QS signals not only serve for intraspecies communications, but are also known to affect other microorganisms and the host (Williams & Camara, 2009). The association between the environment, *P. aeruginosa* QS systems and virulence is schematized in Fig. 1.

Substantial adaptive and genetic changes occur in the genome of *P. aeruginosa* during chronic infections of the CF airways (Foweraker *et al.*, 2005; E. E. Smith *et al.*, 2006; Sriramulu *et al.*, 2005). These changes cause bacteria to diversify and to exhibit characteristics differing from isolates found in the environment outside the body. The adaptation of *P. aeruginosa* during persistent infection of CF lungs can often lead to antimicrobial resistance, alginate overproduction and improved metabolic fitness. Overall, *P. aeruginosa* seems able to adopt a less aggressive profile by repressing its virulence factors and its immunostimulatory products, by growing in biofilms and by metabolically adapting to the microaerobic environment created by airway mucus plugs (Hogardt & Heesemann, 2010). Of these characteristics it appears that the formation of drug-resistant biofilms is strongly associated with the persistence of this bacterium in the CF airways (Høiby *et al.*, 2010; Wagner &

Iglewski, 2008). Also, non-mucoid strains that colonize CF patients can, with time, switch to the mucoid phenotype, show resistance to various antibiotics and become more difficult to eradicate from the airways (George *et al.*, 2009). Other phenotypic variants associated with chronic infections and antibiotic tolerance have also been recovered from the CF airways such as persister cells (Mulcahy *et al.*, 2010) and small-colony variants (Häußler *et al.*, 1999; Schneider *et al.*, 2008). The hypermutability of *P. aeruginosa* CF strains is thought to accelerate the development of antibiotic resistances and the adaptation required for long-term persistence in the host (Maciá *et al.*, 2005).

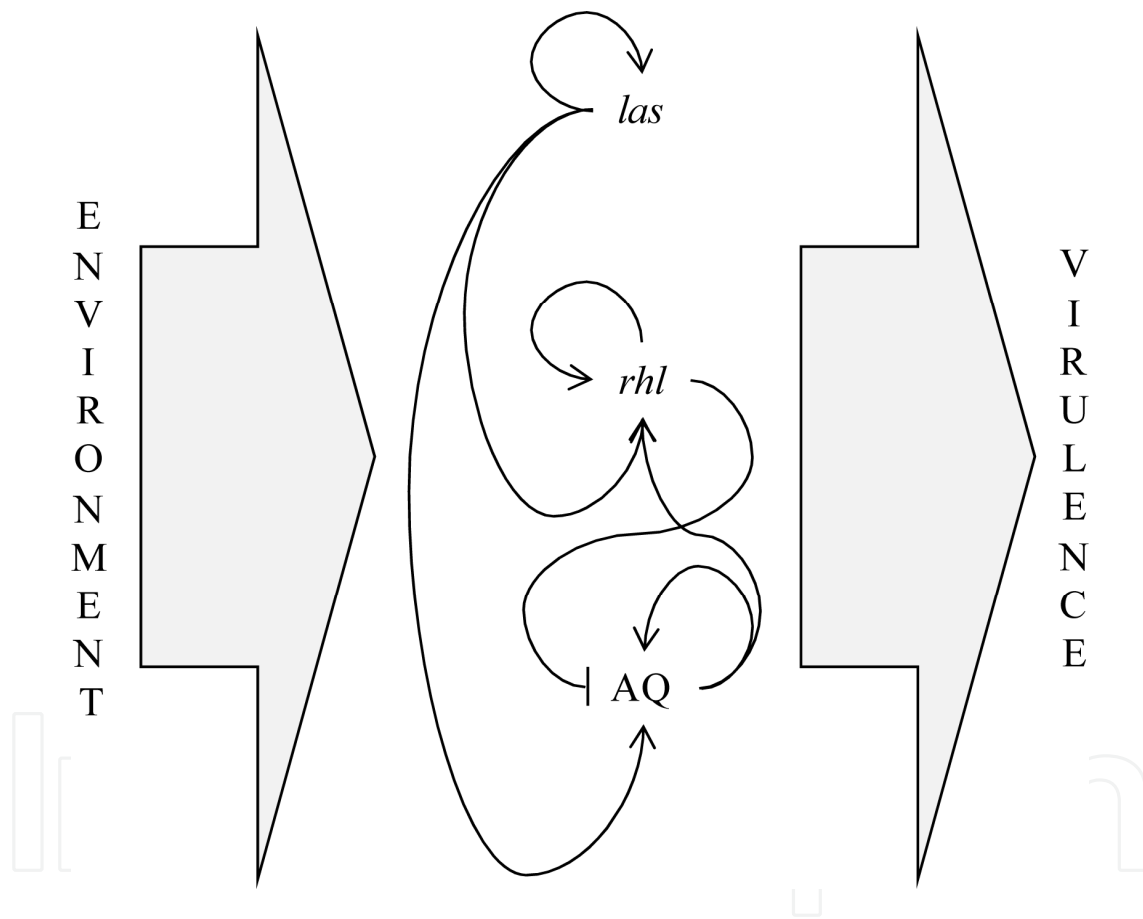


Fig. 1. The virulence of *P. aeruginosa* is controlled by interconnected quorum-sensing (QS) systems that integrate environmental cues and influence virulence gene expression. The two *N*-acylhomoserine lactone systems *las* and *rhl* and the 2-alkyl-4-quinolone (AQ) system are hierarchically interconnected. The *las* system is usually activated first. These QS systems regulate the production of several virulence factors as a function of growth and in response to the environment not only to influence *P. aeruginosa* behavior but also other bacteria of the microbial community as well as host-pathogen interactions.

2.2 Pathogenesis of *S. aureus* in CF

S. aureus can live as a human commensal but also can be an opportunistic Gram-positive pathogen associated with significant mortality in hospitals (Talbot *et al.*, 2006). This bacterium demonstrates an impressive versatility being able to infect several hosts, organs and body sites and cause both life-threatening and chronic infections (Archer, 1998; Goerke & Wolz, 2010). The treatment of *S. aureus* is seriously impeded by antibiotic resistance that has spread among staphylococci and is now being considered as a serious threat to the general population (Witte *et al.*, 2008).

The presence of numerous virulence genes in the genome of *S. aureus* is thought to explain the ability of this bacterium to cause a broad spectrum of diseases (Archer, 1998). The genes involved in pathogenesis are tightly controlled by complex regulatory networks that allow the bacteria to express its virulence factors as a function of the bacterial population density and of its environment (Novick, 2003). One of the most characterized regulatory systems influencing the virulence of *S. aureus* is *agr*, the quorum-sensing accessory gene regulator, that upregulates the production of several extracellular proteins while downregulating many cell-surface proteins (Novick & Geisinger, 2008). Other regulatory networks that govern the expression of accessory genes in *S. aureus* include several two-component regulatory systems and transcription factors such as the alternative sigma factor SigB (Bronner *et al.*, 2004; Novick, 2003). The activity of particular virulence regulators is thought to allow the expression of different sets of factors likely to be required at specific steps during infection or needed for different types of infections.

The contribution of *S. aureus* to the progression of disease in CF is less obvious than that of *P. aeruginosa*. Although the presence of this bacterium in the lower respiratory tract is considered as representative of a pathologic situation, there are still questions concerning the impact of *S. aureus* on the progression of the disease (Lyczak *et al.*, 2002). However, recent data indicate that while the prevalence of *P. aeruginosa* has declined among CF patients over the past few years, the incidence of methicillin-susceptible and methicillin-resistant *S. aureus* (MSSA and MRSA, respectively) has increased in the USA (Razvi *et al.*, 2009). Importantly, detection of persistent MRSA in the respiratory tract of CF patients has been associated with a decrease in survival and with a more rapid decline in lung function (Dasenbrook *et al.*, 2008; Dasenbrook *et al.*, 2010). Furthermore, MRSA bacteria often present a phenotype of mutiresistance to antibiotics (Chambers & Deleo, 2009; Pruneau *et al.*, 2011) and are proficient in biofilm production (Molina *et al.*, 2008).

Notwithstanding these findings, *S. aureus* often persists in the CF lungs for many months or even years and is the cause of recurrent and relapsing infections despite antibiotic treatments (Goerke & Wolz, 2010; B. C. Kahl, 2010). Long-term adaptation of *S. aureus* to the CF environment may occur through mutations (such as those causing the small-colony variant [SCV] phenotype) or through regulatory mechanisms that are still not well understood and that may result in the repression of the *agr* system and establishment of biofilms (Goerke & Wolz, 2010).

SCVs of *S. aureus* are often isolated from chronic infections such as those of the CF airways (Kahl *et al.*, 1998; Moisan *et al.*, 2006; Proctor *et al.*, 2006). The SCV phenotype is frequently caused by either mutations in genes required for electron transport (*e.g.* genes involved in the biosynthesis of hemin or menadione) or by mutations in genes enabling thymidine biosynthesis. Almost all the phenotypic characteristics of SCVs such as the slow growth (*i.e.*, the formation of pin-point colonies when grown on solid media), the altered susceptibility to aminoglycoside antibiotics and the decreased production of exotoxins can be explained by their dysfunctional electron transport (Proctor *et al.*, 2006). Several studies have reported that SCVs are less virulent than prototypical strains *in vivo* yet they can persist as well as the normal strains (Proctor *et al.*, 2006). Our group and others have demonstrated that SCVs are relatively more persistent than their normal counterparts under antibiotic pressure (Bates *et al.*, 2003; Brouillette *et al.*, 2004). A recent study supports the theory that bacterial switching between wild-type and SCV phenotypes is required to sustain chronic infections (Tuchscher *et al.*, 2011).

The hypothesis that SCVs play a role in the development of chronic infections is well supported by *in vitro* experiments demonstrating that these variants have an increased ability to adhere to host tissue components (Mitchell *et al.*, 2008; Vaudaux *et al.*, 2002), to form biofilms (Mitchell *et al.*, 2010a, 2010b; Singh *et al.*, 2009, 2010) and to infect and persist within non-professional phagocytes (Mitchell *et al.*, 2011b; Sendi & Proctor, 2009). These *in vitro* characteristics can be explained by the impact of the defective electron transport chain on the expression of virulence factors which seem to be mostly controlled by the activity of SigB rather than by the *agr* system in SCVs (Moisan *et al.*, 2006; Senn *et al.*, 2005). This altered activation of virulence regulators triggers a sustained expression of several genes encoding cell-surface proteins (*e.g.* the fibronectin-binding protein A *fnbA* gene) and the down-regulation of several exoprotein genes (*e.g.* the hemolysin- α *hla* gene) (Mitchell *et al.*, 2008; Moisan *et al.*, 2006). The sustained expression of *fnbA* has been associated with efficient binding of SCVs to fibronectin (Mitchell *et al.*, 2008). In turn, the formation of a fibronectin bridge between *S. aureus* fibronectin-binding proteins and the $\alpha_5\beta_1$ -integrin of eukaryotic cells (Sinha *et al.*, 1999) probably explains the proficient cellular internalization of SCVs (Sendi & Proctor, 2009). Additionally, the low production of exoproteins and toxins by SCVs is likely to account for their increased persistence within host cells (Sendi & Proctor, 2009). Moreover, the increased production of biofilm by SCVs in comparison with wild-type strains may possibly be explained by the relative influence and the interconnection of the *agr* system and SigB that manipulate the maturation of protein-dependent biofilms (Lauderdale *et al.*, 2009). Furthermore, SCVs have been shown to activate the innate immune response to a lesser extent than that observed with wild-type strains (Tuchscher *et al.*, 2010). This reduced ability of SCVs to induce an inflammatory response may also be attributable to the repression of the *agr* system (Grundmeier *et al.*, 2010) and supports the idea that the SCV phenotype confers on *S. aureus* the ability to remain hidden from the host immune system inside non-professional phagocytes. However, to date, the *in vitro* observations that suggest that the formation of biofilms and the infection of host cells by SCVs allow *S. aureus* to establish long-term infections in CF patients have not been supported by *in vivo* experiments. Further work is required, especially in CF models of pulmonary infections, in order to fully understand the role of SCVs in the pathogenesis of *S. aureus*. Some of the characteristics of the normal and SCV strains are compared in Fig. 2.

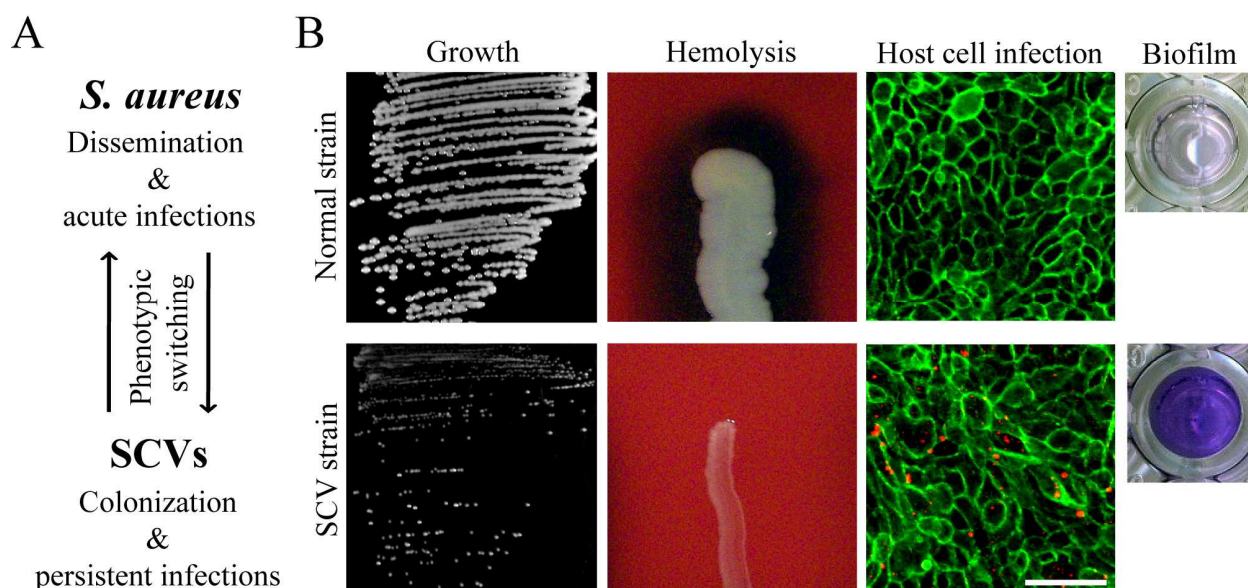


Fig. 2. The ability to switch from the normal to the small-colony variant (SCV) phenotype may have an impact on the virulence of *S. aureus*. (A) *S. aureus* can switch from a normal to a SCV phenotype. The normal and SCV phenotypes are associated with the dissemination of bacteria and acute infections, and with host tissue colonization and persistent infections, respectively. In comparison to normal strains, SCVs form pinpoint colonies, are less or non-hemolytic on blood agar plates and have an increased ability to infect non-professional phagocytes and to form biofilms (B). The host-cell infection pictures were prepared as previously described (Mitchell *et al.*, 2011a, 2011b) and show Calu-3 cells with actin colored in green and internalized *S. aureus* bacteria in red. Scale bar is 50 μm . Biofilm formation was evaluated by crystal violet staining as previously described (Mitchell *et al.*, 2010a, 2010b).

3. Interspecies interactions between CF pathogens

It is now accepted that bacteria can sense signal molecules across species' boundaries and that this signaling influences the development of microbial communities and the virulence and persistence of pathogens during infections (Ryan & Dow, 2008). The clinical impact of polymicrobial infections is receiving more and more recognition from the medical community. There are indeed several examples of polymicrobial infections where at least two different microorganisms influence the course of the disease by synergistic, additive or antagonistic effects. Also, biofilms are thought to be of major importance for the pathogenesis of bacteria in the context of CF lung infections (Davies & Bilton, 2009) and can be considered as integrated and complex polymicrobial communities whose development is controlled by interspecies communications (Stoodley *et al.*, 2002). Accordingly, and as previously underlined, infections of the CF airways is highly polymicrobial and there is growing evidence that many of these microorganisms interact (Sibley & Surette, 2011) as observed between *P. aeruginosa* and *S. aureus* (Biswas *et al.*, 2009; Hoffman *et al.*, 2006; Mashburn *et al.*, 2005; Mitchell *et al.*, 2010b; Qazi *et al.*, 2006; Yang *et al.*, 2011), *P. aeruginosa* and *Burkholderia* spp. (Bakkal *et al.*, 2010; Chatteraj *et al.*, 2010; Riedel *et al.*, 2001; Weaver & Kolter, 2004), *P. aeruginosa* and *S. maltophilia* (Ryan *et al.*, 2008), *P. aeruginosa* and *C. albicans* (McAlester *et al.*, 2008), and more generally with a large proportion of the organisms found in the CF airways (Duan *et al.*, 2003; Sibley *et al.*, 2008).

3.1 Interactions between *P. aeruginosa* and *S. aureus*

Given that *P. aeruginosa* and *S. aureus* are highly prevalent and commonly co-isolated from the CF airways (Harrison, 2007; Hoffman *et al.*, 2006), a great deal of effort has been directed toward the characterization of the interaction between them. Historically, synergistic interactions between these two species have been proposed and it was thought that *S. aureus* could sensitize the lungs for subsequent infections by *P. aeruginosa* (Lyczak *et al.*, 2002). However, it is now clear that antagonistic interactions also exist between these bacteria as *P. aeruginosa* has the ability to provoke lysis of *S. aureus* cells (Mashburn *et al.*, 2005; Palmer *et al.*, 2005). The structures of some *P. aeruginosa* exoproducts that are known to impact on *S. aureus* are shown in Fig. 3.

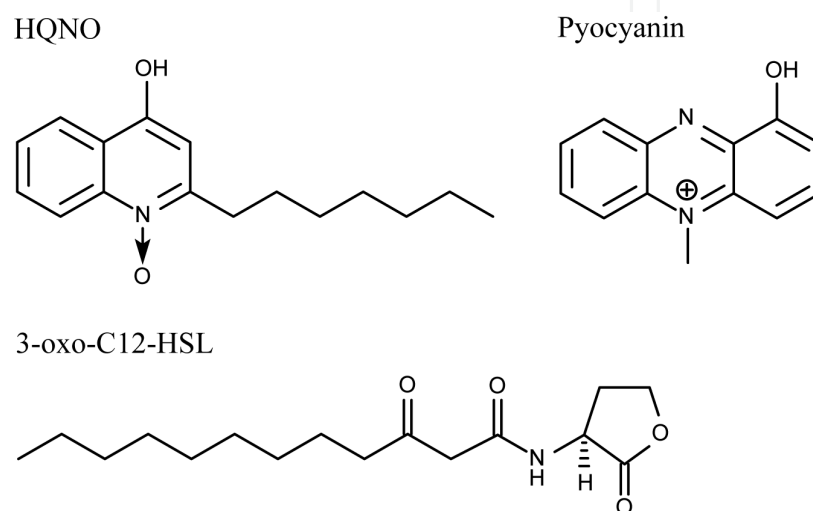


Fig. 3. Structure of *P. aeruginosa* molecules influencing *S. aureus* viability and pathogenesis.

The antistaphylococcal activities of pseudomonal HQNO (see Fig. 3) and other 4-hydroxy-2-alkylquinolines (HAQs) have been known for many years and are molecules that can generally suppress the growth of many Gram-positive bacteria (Lightbown & Jackson, 1954; Machan *et al.*, 1992). Mashburn *et al.* (2005) suggested that *P. aeruginosa* could exploit this property to lyse *S. aureus* cells in order to use the released iron for growth in low-iron environments. Interestingly, HQNO also allows some Gram-positive to grow slowly in presence of aminoglycoside antibiotics (Lightbown, 1954) and the reason for this has remained a mystery for several years. The protection provided by HQNO against the inhibitory activity of aminoglycosides was finally found to be related to the ability of this molecule to inhibit the Gram-positive electron transport chain (Hoffman *et al.*, 2006), which is required for aminoglycoside uptake (Bryan & Van Den Elzen, 1977). It was further shown that prolonged exposure of *S. aureus* to HQNO (or to *P. aeruginosa*) selects for SCVs (Hoffman *et al.*, 2006). We subsequently demonstrated that HQNO produced by *P. aeruginosa* not only stimulates the formation of *S. aureus* biofilms but also modulates the activity of virulence regulators. More particularly, while increasing the activity of SigB and the expression of *sarA*, HQNO downregulates the expression of the effector of the *agr* system (RNAlII). This modulation of regulator activities is likely to influence the expression of several virulence factors as was shown for *fnbA* and *hla* (Mitchell *et al.*, 2010b). The interaction of PQS with the cell envelope of *P. aeruginosa* is also known to trigger the release of membrane vesicles (MVs) which contain toxins, DNA, antimicrobials as well as HHQ, PQS and HQNO. It is thought that MVs are

important for the trafficking of PQS within the *P. aeruginosa* population as PQS is poorly soluble in water. However, since MVs contain AQS including HQNO, they could also inhibit staphylococcal growth (Mashburn & Whiteley, 2005). Furthermore, *P. aeruginosa* produces other small-molecule respiratory inhibitors such as pyocyanin (see Fig. 3) and hydrogen cyanide which can affect the respiration of *S. aureus* (Voggu *et al.*, 2006) and potentially select for the SCV phenotype (Biswas *et al.*, 2009). Whether and how these other respiratory inhibitors influence the virulence of *S. aureus* remains to be determined. Fig. 4 shows the effect of *P. aeruginosa* on the growth of *S. aureus*, the HQNO-mediated emergence of SCVs and the stimulation of *S. aureus* biofilm production by HQNO.

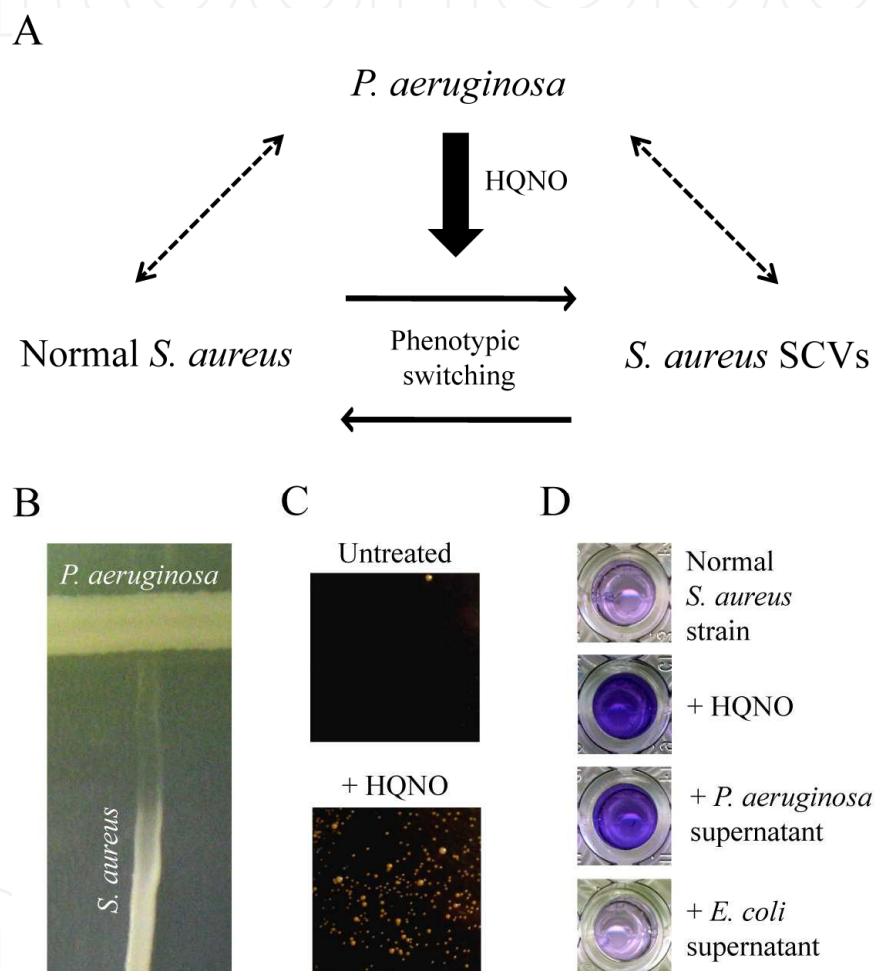


Fig. 4. *P. aeruginosa* influences the phenotypic switching of *S. aureus* and may have an impact on its virulence. (A) *P. aeruginosa* promotes the emergence of SCVs from normal *S. aureus* strains through the production of respiratory inhibitors such as HQNO and pyocyanin. Dotted arrows indicate other potential interactions between these bacterial species. (B) *P. aeruginosa* inhibits the growth of *S. aureus*. (C) HQNO stimulates the emergence of SCVs in *S. aureus*. Bacteria were treated or not with 10 $\mu\text{g}/\text{ml}$ of HQNO for 18 h and plated on agar containing gentamicin at 4 $\mu\text{g}/\text{ml}$ to reveal the presence of SCVs. (D) HQNO and *P. aeruginosa* culture supernatants enhance biofilm production by a normal *S. aureus* strain. *S. aureus* biofilms were produced in the presence of 10 $\mu\text{g}/\text{ml}$ of HQNO or in the presence of culture supernatants from *P. aeruginosa* or *Escherichia coli* (acting as a negative control) and were revealed by crystal violet staining as previously described (Mitchell *et al.*, 2010a, 2010b).

It also appears that factors other than pseudomonal respiratory inhibitors are involved in the microbial interactions occurring between *P. aeruginosa* and *S. aureus*. Importantly, most of the antistaphylococcal activity found in *P. aeruginosa* culture supernatant is attributed to the staphylolytic endopeptidase LasA (Kessler *et al.*, 1993). Qazi *et al.* (2006) demonstrated that some long-chain 3-oxo-substituted *N*-acylhomoserine lactones (AHL) such as 3-oxo-C12-homoserine lactone (see Fig. 3) can inhibit the growth of *S. aureus* as a function of their concentrations. The 3-oxo-C12-HSL also modulates the production of *S. aureus* exotoxins and cell-surface proteins through the repression of *sarA* and *agr* by interaction with a specific and saturable receptor(s) at the cytoplasmic membrane. Furthermore, the 3-oxo-C12-HSL is capable of undergoing internal re-arrangement to form the 3-oxo-C12-tetrameric acid, which also has an inhibitory activity against Gram-positive bacteria (Kaufmann *et al.*, 2005). A recent study investigated the formation of biofilms by *P. aeruginosa*-*S. aureus* co-cultures using a flow chamber system and confocal microscopy (Yang *et al.*, 2011). This study demonstrated that wild-type *P. aeruginosa* facilitates *S. aureus* microcolony formation, but that *mucaA* and *rpoN* mutants do not have this property and tend to outcompete *S. aureus*. A role for type IV pili in this phenomenon was proposed to occur through binding of extracellular DNA, and it was demonstrated that *P. aeruginosa* protects *S. aureus* against *Dictyostelium discoideum* phagocytosis when in co-culture biofilms.

Some studies have been carried out to substantiate the interactions between *P. aeruginosa* and *Staphylococcus epidermidis*, a bacterium closely related to *S. aureus*. It was shown that some *P. aeruginosa* extracellular products (possibly polysaccharides) provide a competitive advantage over *S. epidermidis* (Qin *et al.*, 2009). Furthermore, Pihl *et al.* (Pihl *et al.*, 2010a, 2010b) demonstrated that some *S. epidermidis* strains were better fitted than others to coexist in biofilms with *P. aeruginosa* whereas the ability of *P. aeruginosa* to inhibit *S. epidermidis* biofilms varied between clinical isolates. These authors suggested that specific *P. aeruginosa* strains might be selected during infections to counteract chronic colonization by *S. epidermidis* in order to allow the persistence and dominance of *P. aeruginosa*. Whether the genetic background of each strain also influences interspecies interactions between *S. aureus* and *P. aeruginosa* and whether the outcome of these interactions varies in each CF patient remains to be determined. Recent studies, which demonstrate that *P. aeruginosa lasR* mutants are frequently found in CF, indicate that some of the abilities of this bacterium to influence the virulence of *S. aureus* may indeed be lost during CF lung infections (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2009).

Other questions remain open. For example, the real impact of *S. aureus*-*P. aeruginosa* co-infections in CF is not known and convincing clinical data as well as co-infection in adequate experimental models are clearly missing. Whether *S. aureus* and *P. aeruginosa* have a synergistic or antagonistic effect on the progression of the disease is not known and may potentially be influenced by the nature of each clinical isolate. Furthermore, whereas the effect of *P. aeruginosa* on *S. aureus* has been studied widely, almost no investigations address the potential effect of *S. aureus* on the virulence of *P. aeruginosa*. Interestingly, Korgaonkar and Whiteley (2011) proposed a model in which *P. aeruginosa* senses surrounding bacteria by monitoring the presence of exogenous peptidoglycan and responds to it by increasing the production of the virulence factor pyocyanin.

4. New therapeutic approaches in CF

Humanity is now facing a post-antibiotic era defined by its limited capability to combat microbial infections caused by antibiotic resistant pathogens. The increased number of nosocomial and community-acquired infections caused by microorganisms resistant to at least two classes of conventional antibiotics is becoming an important public health problem. The global rise of antimicrobial resistance combined with the slow discovery and approval processes for classical antibiotics has resulted in the present urgent need for new and innovative therapeutic approaches. One of the reasons for multidrug resistance is that current antibiotics were designed around a limited number of chemical scaffolds with few major modifications since the 1980s. This has left plenty of opportunities for antibiotic resistance mechanisms to develop and spread worldwide (Shah, 2005; Talbot *et al.*, 2006). The identification of new antimicrobial targets and the development of novel therapeutic approaches may be facilitated by a better understanding of bacterial pathogenesis both at the single species and bacterial community levels. Antibiotic resistant bacteria are often recovered from CF patients but recalcitrance to antibiotic therapies is not only caused by bacterial genes that encode conventional mechanisms of antibiotic resistance. Indeed, the ever changing epidemiology of the CF airways (Razvi *et al.*, 2009) and the acquisition of bacterial phenotypes inherently resistant to antimicrobials contribute to treatment failures. Although some antibiotics have recently been approved or are close to being approved for clinical use and are promising in the context of CF (Parkins & Elborn, 2010), this section will talk about some novel therapeutic approaches that take into account the pathogenesis and the adaptation of CF pathogens in the context of polymicrobial infections. However, in order to appreciate the entirety of the efforts directed toward the cure of CF, it should be kept in mind that a number of strategies other than those using antibiotherapies are also being considered (George *et al.*, 2009).

4.1 Modulation of biofilm-forming microbial communities

The formation of biofilms by CF pathogens is thought to be a major virulence asset that promotes resistance both to antimicrobials and the host immune system (Costerton *et al.*, 1999; Davies & Bilton, 2009; Høiby *et al.*, 2010; Stewart, 2002). This fully justifies current research aimed at the development of therapeutic strategies that target biofilm formation and dispersal (Simões, 2011). Current management practices for *P. aeruginosa* infections include hygienic measures (Høiby & Pedersen, 1989), early aggressive eradication by antimicrobial therapy (Döring & Høiby, 2004), the use of nebulized DNase (Frederiksen *et al.*, 2006) and chronic suppressive antibiotic therapy (Bjarnsholt *et al.*, 2009; Döring *et al.*, 2000). However, even if these methods are undeniably successful to a certain extent, chronic infection ultimately occurs and a gradual increase in the level of resistance is observed (Ciofu *et al.*, 1994). New therapeutic approaches specifically targeting biofilms should thus be useful in the context of CF lung infections and should decrease the occurrence or development of antibiotic resistance.

Most natural biofilms are polymicrobial (Stoodley *et al.*, 2002) and the polymicrobial nature of CF lung infections needs to be considered in novel therapeutic approaches (Sibley *et al.*, 2009; Sibley & Surette, 2011). Whereas some microbes may predispose the tissue toward the colonization by others, there may also be competition among bacterial populations and the removal of one pathogen could create an opportunity for another to expand (Harrison,

2007). On the other hand, tampering with the CF airway microbial community may lead to more effective treatment of chronic infections (Moore *et al.*, 2005). Some data even suggest that a healthy gut microflora protects against some respiratory pathogens (Alvarez *et al.*, 2001; Villena *et al.*, 2005) and that care should be taken not to deplete the gut microflora when oral or intravenous routes of antibiotic administration are used. It is also conceivable that monitoring the population dynamics of polymicrobial infections can be used to predict the efficacy of antimicrobial therapy and to optimize treatments (Rogers *et al.*, 2010). As such, the impact of antimicrobial chemotherapies on microbial communities should be assessed to detect unwanted effects. As an example, aminoglycosides, which are indicated for the management of acute exacerbations, the control of chronic infections and the eradication of recently acquired *P. aeruginosa*, have also been shown to induce bacterial biofilms in both *P. aeruginosa* (Hoffman *et al.*, 2005) and *S. aureus* (Mitchell *et al.*, 2010a).

The next sections provide examples of methods by which biofilm infections may be potentially overcome using different strategies that include targeting specific microbial phenotypes, influencing the pathogenesis of bacteria through the manipulation of cell-to-cell signaling and the enhancement of preexisting antimicrobial therapies against persistent forms of bacteria.

4.2 Targeting the persistent microbial phenotype

Bacteria often encounter unfavorable conditions during infection that limit bacterial growth and oblige the microorganisms to enter a quiescent state in order to persist within the host (Kolter *et al.*, 1993; Nataro *et al.*, 2000). Dormant bacteria are well-known for their tolerance to antibiotics normally active against rapidly dividing cells and often require prolonged periods of treatment (Coates *et al.*, 2002; Neu, 1992). The highly refractory nature of biofilms to eradication by chemotherapy is thought to be at least partly attributable to the presence of metabolically inactive cells (Fux *et al.*, 2005). The inefficacy of antibiotics against non-multiplying bacteria thus results in slow or partial death, prolongs the duration of therapy and increases the emergence of genotypic resistances. Accordingly, targeting slow-growing or non-dividing bacteria should provide substantial therapeutic benefits.

Membrane-acting agents are usually active against bacteria in all their phases of growth and are thus good candidates for the development of antimicrobials that target slow-growing and non-dividing bacteria. As an example, the novel porphyrin antibacterial agents XF-70 and XF-73 were shown to remain highly active against this type of bacteria (Ooi *et al.*, 2010). Hu *et al.* (2010) found that the small quinolone-derived compound HT61 was active against non-multiplying MSSA and MRSA by causing depolarization of the cell membrane and destruction of the cell wall. Antimicrobial peptides also interact and permeabilize the bacterial membrane and there is a good probability that they act on slow-growing bacteria that form biofilms (Batoni *et al.*, 2011). Bioactive peptides may even have additional benefits for CF therapeutic applications due to their anti-inflammatory and immunomodulating activities (Scott *et al.*, 2007; Zhang *et al.*, 2005).

The resistance of biofilms to killing by most antimicrobial agents is thought to be more specifically attributable to the presence of non-dividing persister cells (Lewis, 2007). Persisters are dormant bacteria that present a global slowdown in metabolic processes, do not divide and are tolerant to antibiotics. In other words, they have the ability to survive the

effects of antibiotics without the use of drug-specific resistance mechanisms. Persisters have been described for *S. aureus* (Allison *et al.*, 2011; Singh *et al.*, 2009) and *P. aeruginosa*, with a recent study that shows the emergence of strains producing high levels of persister cells in CF patients (Mulcahy *et al.*, 2010). Currently, there are only a few therapeutic strategies that are considered for targeting persister cells. One such is the combination of conventional antibiotics with an inhibitor of an essential persister protein (Lewis, 2007). Also, repeated- or pulse-dosing of antibiotics could allow persister cells to resuscitate in order to be killed by subsequent antibiotic administration. The development of specific pro-antibiotics which could irreversibly bind to bacterial targets is also being considered (Lewis, 2007). An outstanding recent study shows that the use of specific metabolic stimuli enables the killing of persister cells with aminoglycoside antibiotics by modulating the proton-motive force required for the uptake of these drugs. The proof of concept for the latter approach has been demonstrated against biofilms and also in a model of chronic infection (Allison *et al.*, 2011).

As we have previously mentioned, SCVs are often associated with relapsing and persistent infections. In addition to the increased ability of these variants to form biofilms (Al Laham *et al.*, 2007; Häußler *et al.*, 2003; Mitchell *et al.*, 2010a, 2010b; Singh *et al.*, 2009, 2010; von Götz *et al.*, 2004), SCVs are well-known for their ability to infect and persist within non-professional phagocytes (Sendi & Proctor, 2009) and there is a limited choice of antibiotics able to act against intracellular bacteria. Nguyen *et al.* (Nguyen *et al.*, 2009a) reported a considerable decrease in the efficacy of most antibiotics against intracellular SCVs in comparison to that seen against extracellular bacteria, but, most importantly, in comparison to their efficacy against the normal-phenotype bacteria. Nevertheless, the authors noted that four antibiotics (quinupristin-dalfopristin, moxifloxacin, oritavancin and rifampicin) were more effective in killing intracellular SCVs. In addition, we recently described the first known molecule to specifically target the SCV phenotype of *S. aureus* (Mitchell *et al.*, 2011a). Tomatidine (TO) is the aglycone form of the plant secondary metabolite tomatine. The structure and the main biological activities of TO against *S. aureus* are presented in Fig. 5A. We found that TO has a bacteriostatic activity against SCVs, but not against normal strains. More importantly, we showed that TO has the ability to inhibit the replication of SCVs internalized in CF-like human airway epithelial cells (Mitchell *et al.*, 2011a). The specificity of the action of TO against SCVs was linked to the dysfunctional electron transport chain of these variants. Accordingly, HQNO sensitized normal *S. aureus* strains to TO (see Fig. 5B), which suggests that TO may be especially effective in the context where *P. aeruginosa* and *S. aureus* co-infect a CF patient. Although TO causes a marked inhibition of protein synthesis in bacteria showing a dysfunctional electron transport chain, the exact mechanism of action of TO on SCVs remains to be elucidated. Other biological activities for TO are discussed below.

4.3 Targeting virulence

Another emerging concept in the development of novel therapeutic approaches is the possibility to modulate the expression of virulence factors that are thought to be of major importance in the establishment of a particular infection. Modulators or blockers of pathogenesis are particularly interesting because it is speculated that, since they do not inhibit growth or kill bacteria, their use will not yield a strong selective pressure for resistance development. In this context, most attention has been directed toward the interference of bacterial QS and cell-to-cell signaling to inhibit virulence or biofilm

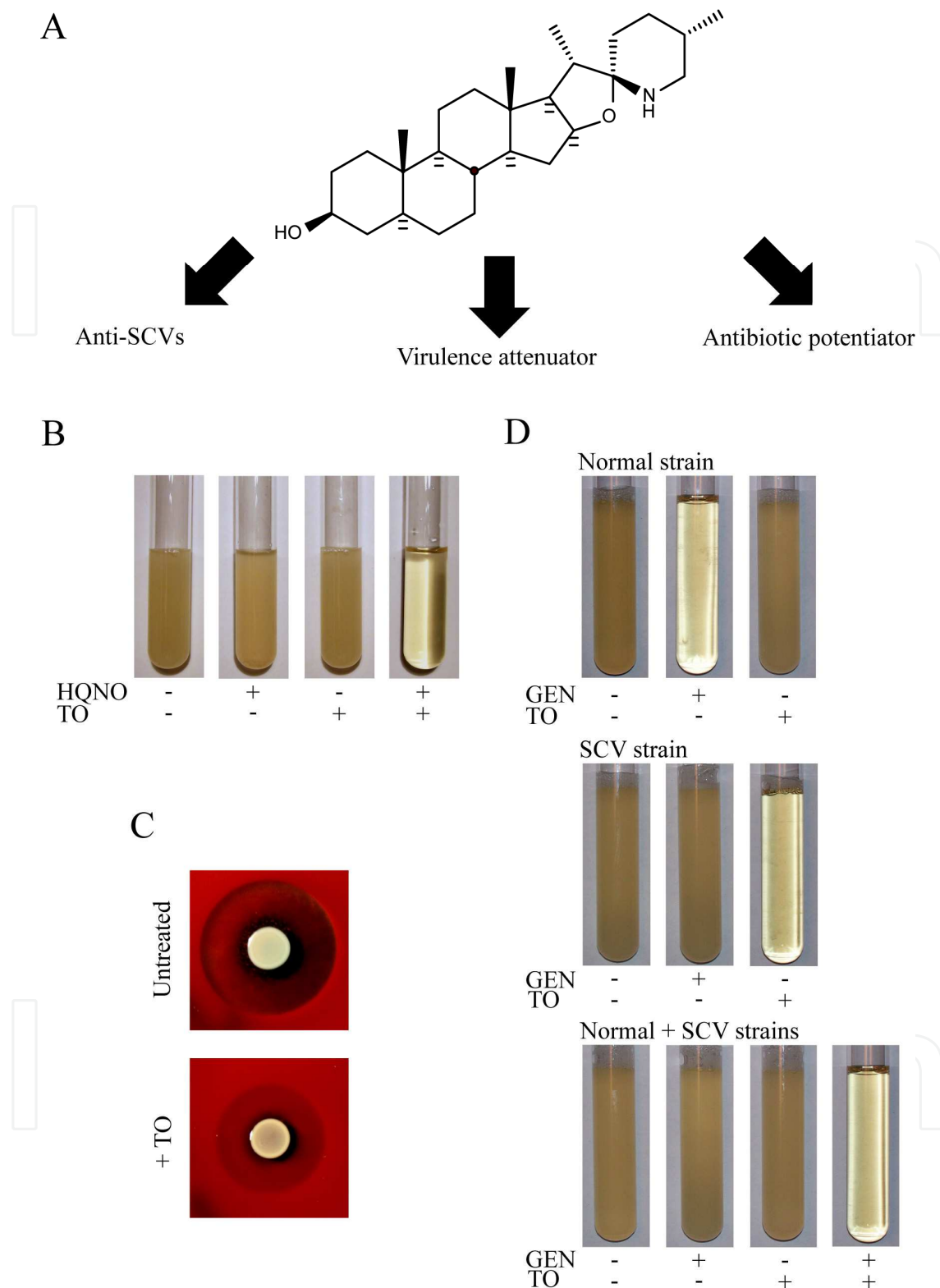


Fig. 5. Biological activities of tomatidine against *S. aureus*. (A) Tomatidine (TO) is a steroidal alkaloid molecule that inhibits both the extracellular and intracellular replication of SCVs, represses the expression of several *agr*-regulated exoproducts and potentiates the bactericidal activity of aminoglycoside antibiotics against prototypical *S. aureus*. (B) TO (8 $\mu\text{g}/\text{ml}$) inhibits the growth of a normal *S. aureus* strain in presence of HQNO (20 $\mu\text{g}/\text{ml}$).

Bacteria were inoculated at 10^5 - 10^6 CFU/ml and incubated 24 h at 35°C with shaking. (C) TO (8 µg/ml) inhibits the hemolytic ability of a normal-growing *S. aureus* strain. (D) TO may be used in combination with aminoglycosides (e.g., gentamicin [GEN]) to eradicate a population of *S. aureus* composed of normal and SCV bacteria. Bacteria were inoculated at 10^5 - 10^6 CFU/ml and incubated 24 h at 35°C with shaking in presence of 4 µg/ml of GEN and/or 0.12 µg/ml of TO. The clear test tubes show no bacterial growth.

formation (Njoroge & Sperandio, 2009; Rasko & Sperandio, 2010). However, although targeting the QS systems of *P. aeruginosa* in CF infections may increase the susceptibility of biofilms to clearance by antibiotics (Hentzer *et al.*, 2003), interference with the QS *agr* system of *S. aureus* may not be a good strategy since it could increase the formation of biofilms and increase bacterial persistence (Novick & Geisinger, 2008; Otto, 2004). The discovery of compounds that attenuate or abolish the cross talk between the QS systems of different bacterial species may be more promising. It remains to be determined whether interference with interspecies communications has the potential to decrease the overall virulence or the cohesion of polymicrobial communities and more particularly of those found in CF.

Several plant products have been shown to act as “virulence attenuators” of human pathogens (González-Lamothe *et al.*, 2009). Virulence attenuators modulate the virulence or the ability of the bacterium to adapt to the host environment. This gives a competitive advantage to the host immune system. As an example, a garlic extract was shown to interfere with the QS of *P. aeruginosa*, to sensitize its biofilm to tobramycin treatments and to improve clearance of bacteria in a pulmonary mouse model (Bjarnsholt *et al.*, 2005; Rasmussen *et al.*, 2005). Ginseng extract was also shown to alter the virulence of *P. aeruginosa* by interfering with QS, destroying biofilms, promoting phagocytosis by airway phagocytes and by protecting animals from the development of chronic lung infections (Song *et al.*, 1997a, 1997b, 2010; Wu *et al.*, 2011). Some of our own transcriptional analyses of bacteria exposed to plant products have shed light on the effect of TO on the expression of virulence factors by normal *S. aureus* strains (Bouarab *et al.*, 2007). We demonstrated that TO causes a repression in the expression of many extracellular toxins and of RNAPIII, the effector molecule of the *agr* system, and thus it inhibits the hemolytic activity of *S. aureus*. We further showed that TO inhibits biofilm formation by *S. aureus* SCVs, probably through the induction of bacteriostasis (Mitchell *et al.*, 2009). We suggest that the overall negative effects of TO on the virulence and the growth of both normal and SCV *S. aureus* strains could be used in the management of both acute and chronic lung infections in CF patients. Fig. 5C shows the inhibitory effect of TO on the hemolytic ability of a normal *S. aureus* strain.

Other studies have also promoted the use of virulence factor-based therapies against *S. aureus*. For example, QS autoinducing peptide variants were shown to inhibit heterologous *agr* activation and were proposed as therapeutic agents (Novick & Geisinger, 2008). Also, a blocker of the synthesis of staphyloxanthin, the golden-carotenoid pigment of *S. aureus* that promotes resistance to reactive oxygen species and host neutrophil-based killing, increased the susceptibility of *S. aureus* to killing by human blood and the innate immune clearance in a mouse infection model (Liu *et al.*, 2008). Other researchers have attempted to achieve virulence attenuation by manipulation of bacterial metabolism (Lan *et al.*, 2010; Zhu *et al.*, 2009). Another possible approach is to target the bacterial pathways for programmed cell death which have been identified in several species (Engelberg-Kulka *et al.*, 2004).

4.4 Enhancing preexisting antimicrobial therapies

The use of synergistic combinations of antimicrobial compounds is an old strategy that continues to be tantalizing especially against the polymicrobial populations found in the CF airways. Accordingly, Høiby (2011) suggests that the effectiveness of combination therapies should be tested in the context of CF lung infections. Several combinations of old antibiotics indeed showed promising synergistic effects *in vitro* and/or *in vivo* such as fosfomycin-tobramycin, tobramycin-colistin and ciprofloxacin-colistin combinations, with the ciprofloxacin-colistin combination thought to be efficient even against *P. aeruginosa* biofilms (Høiby, 2011). A large screen of double and triple antibiotic combinations was tested on biofilm-grown *B. cepacia* and *P. aeruginosa* in order to identify effective antibiotic combinations for the treatment of CF patients (Dales *et al.*, 2009). Combinations of antibiotics may also be useful in order to improve the efficiency of antimicrobial treatments against intracellular SCVs. As an example, some drug combinations that included rifampicin were most effective against intracellular *S. aureus* of both normal and SCV phenotypes. (Baltch *et al.*, 2008). Nguyen *et al.* (2009b) also showed additive or synergistic effects between oritavancin, moxifloxacin and rifampicin against intracellular SCVs.

The combination of antimicrobial agents with non-antibiotic compounds is also an attractive approach (George *et al.*, 2009). Several plant products are “antibiotic potentiators” that can act as bacterial efflux pump inhibitors, cell wall-acting agents or membrane destabilizing agents to provide synergy with conventional antibiotics (González-Lamothe *et al.*, 2009). Interestingly, we have recently demonstrated that TO potentiates the bactericidal activity of aminoglycoside antibiotics against normal *S. aureus* strains of diverse clinical origins and antibiotic susceptibility patterns (unpublished results). Although the mechanism(s) by which this effect occurs is yet unknown, TO may prove useful in combination therapy with aminoglycoside antibiotics in the treatment of CF lung infections as exemplified in Fig. 5D. According to Mohtar *et al.* (Mohtar *et al.*, 2009), a vast number of other plant products with antimicrobial activity await discovery.

5. Conclusion

Complex polymicrobial communities colonize the CF airways and interspecies interactions are likely to play a role in the course of respiratory infections. *P. aeruginosa* and *S. aureus* are prevalent pathogens often simultaneously found in CF patients and for which microbial interactions that modulate virulence are already well documented. In communities and by using intraspecies and interspecies cell-to-cell communication, these pathogens have the ability to form biofilms and to adopt persistent phenotypes such as persister cells and SCVs. These phenotypes confer non-specific resistance to antimicrobials and to the host immune system. The development of novel therapeutic approaches that take into account polymicrobial communities and the various strategies that bacteria have elaborated to adapt and persist within the CF airways should help to eradicate chronic and life-threatening infections in CF.

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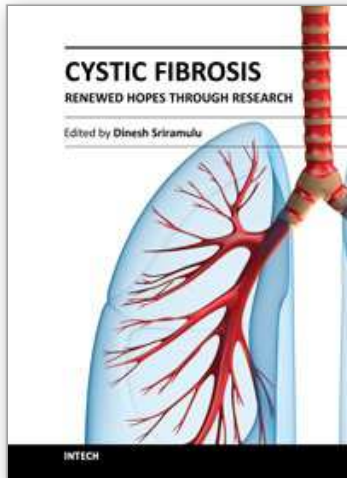
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Living healthy is all one wants, but the genetics behind creation of every human is different. As a curse or human agony, some are born with congenital defects in their menu of the genome. Just one has to live with that! The complexity of cystic fibrosis condition, which is rather a slow-killer, affects various organ systems of the human body complicating further with secondary infections. That's what makes the disease so puzzling for which scientists around the world are trying to understand better and to find a cure. Though they narrowed down to a single target gene, the tentacles of the disease reach many unknown corners of the human body. Decades of scientific research in the field of chronic illnesses like this one surely increased the level of life expectancy. This book is the compilation of interesting chapters contributed by eminent interdisciplinary scientists around the world trying to make the life of cystic fibrosis patients better.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

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