MINIREVIEW

The role of quorum sensing in chronic cystic fibrosis *Pseudomonas aeruginosa* infections

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Abstract

Studies on cultured cells and in infection models have shown that cell density-dependent quorum-sensing (QS) controls many of the known virulence factors of *Pseudomonas aeruginosa*. However, it is less clear what role QS plays in chronic human lung infections associated with cystic fibrosis (CF). The involvement of QS in biofilm development, crucial to the establishment of long-term infections, suggests a role in the early stages of infection. However, the accumulation of QS mutants during chronic CF infections has been taken to indicate that any role diminishes thereafter. Here, we discuss the evidence for a continuing role for QS in *P. aeruginosa* CF infections, including QS activity in CF sputa and CF-relevant effects of QS-regulated products, such as pyocyanin. Bacterial population behaviour in CF is complex, and the exact roles of QS remains unclear. Therapeutic strategies directed against QS suggest that a greater understanding of bacterial populations during infection would be a valuable research goal from a clinical perspective.

Introduction

Quorum-sensing (QS) circuits are complex, especially in *Pseudomonas aeruginosa*, where multicomponent communication and regulatory network interactions operate. It is clear from studies in infection models that the QS systems of *P. aeruginosa* control the production of factors implicated in virulence. Often researchers seek a justification for their work by suggesting a key role for QS-regulated virulence factors in *P. aeruginosa* infections, especially in cystic fibrosis (CF), thus linking the need for a greater understanding of QS regulation with the development of novel therapeutic strategies. However, the apparent accumulation of QS mutants during chronic infections in CF may indicate that any role for QS is limited, or restricted to the early stages. In this review, we consider the evidence for an ongoing role for QS throughout the life-long chronic infections of CF patients.

Pseudomonas aeruginosa in CF

CF is the most common life-shortening inherited disease among Caucasian humans. It results from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a protein involved in the transport of electrolytes across epithelial and other cell membranes. The defect leads to highly viscous mucous and a failure in the airway mucociliary clearance mechanisms. Hence, the normally sterile lower airways are compromised by the accumulation of mucous and trapped bacteria (Hart & Winstanley, 2002). The most common and important CF pathogen is *P. aeruginosa*, which causes chronic pulmonary infections. The establishment of *P. aeruginosa* infection in CF is a clear predictor of morbidity and eventual mortality. Because of the ability of *P. aeruginosa* to switch to a mucoid phenotype and adopt a biofilm lifestyle, after which the infection is never cleared, initial colonization is a key stage. Early and aggressive therapy can prevent the establishment of infections. However, most CF patients do eventually become infected with *P. aeruginosa*.

With its large genome, *P. aeruginosa* is extremely versatile and adaptable. In humans *P. aeruginosa* is an opportunist pathogen, causing infections of various parts of the human body, including the eye, burns and other wounds, as well as the CF respiratory tract. For an opportunist pathogen, *P. aeruginosa* carries an impressive array of virulence factors
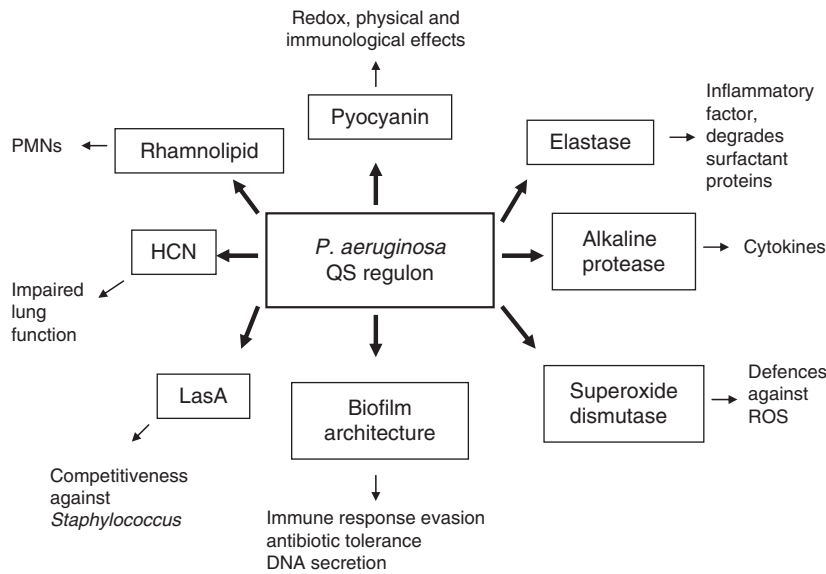
Fig. 1. *Pseudomonas aeruginosa* QS-regulated virulence factors and their relevance to CF. ROS, reactive oxygen species; PMN, polymorphonuclear leukocyte (neutrophil).

including a number of potent secreted toxins, many of which are under the control of the QS regulatory system (Figs 1 and 2).
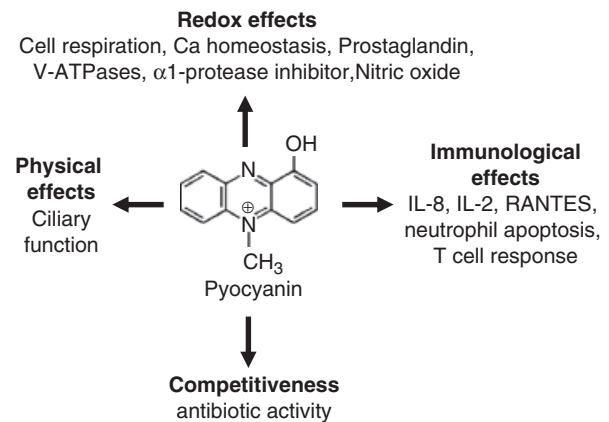
QS in *P. aeruginosa*

Since the first reports of cell density-dependent bioluminescence in the fish symbiont *Vibrio fischeri*, bacterial QS regulatory systems have caught the imagination of microbiologists. Communication occurs via the signalling molecules homoserine lactones (HSLs), which are secreted, accumulate in the external environment and, on reaching a critical concentration, are sensed by bacteria, triggering various responses. The *P. aeruginosa* QS network is especially interesting because of its complexity, with two interdependent LuxIR-type QS systems, LasIR and RhlIR, interacting with a quinolone signal and numerous regulators and sigma factors. The QS regulatory network of *P. aeruginosa* has been reviewed recently (Girard & Bloemberg, 2008) and will not be discussed in detail here. Techniques such as microarray transcriptomics have been used to identify the extent of the QS regulon, which comprises > 5% of the *P. aeruginosa* genome. QS mutants are attenuated for virulence in various infection models including the burned mouse model (Cao *et al.*, 2001), mouse pneumonia model (Tang *et al.*, 1996), rat model (Potvin *et al.*, 2003), *Arabidopsis thaliana* (Rahme *et al.*, 2000), *Caenorhabditis elegans* (Tan & Ausubel, 2000) and *Dictyostelium discoideum* (Cosson *et al.*, 2002).

QS and biofilm formation

Biofilm formation is an important feature of *P. aeruginosa* pathogenicity and contributes significantly to the resistance of *P. aeruginosa* to aggressive antimicrobial therapy (Stewart & Costerton, 2001). In the laboratory *P. aeruginosa* can form two types of biofilms, termed 'flat' and 'structured', respectively. It has been shown that alginate-overproducing strains exhibit a highly structured architecture and are significantly more resistant to the antibiotic tobramycin (Hentzer *et al.*, 2001), linking the CF signature mucoid phenotype directly with biofilm formation. The question of whether there is direct involvement of the QS system in biofilm formation has been a key issue for some time (reviewed by Kirisits & Parsek, 2006). Studies using *las* or *rhl* mutants do suggest a role for QS in biofilm development, but interstrain variations, subtle changes in gene expression due to environmental factors, and putative interactions at various stages in biofilm formation have all contributed to a lack of clarity. However, in a mouse infection model, QS inhibitory drugs reduce biofilm formation (Christensen *et al.*, 2007).

Fig. 2. Effects of pyocyanin.

It has been suggested that the airway mucosa in CF is an anaerobic environment. There is evidence suggesting that QS plays a role in the viability of *P. aeruginosa* anaerobic biofilms (Hassett *et al.*, 2002). Nevertheless, the precise role of QS in biofilm formation in CF remains unclear.

Pyocyanin in CF

Phenazines, nitrogen-containing heterocyclic compounds synthesized by *Pseudomonas* species and a number of other bacteria, are secondary metabolites with a number of important biological activities. Pyocyanin (5-methyl-1-hydroxyphenzine) is a blue, redox-active phenazine secreted by *P. aeruginosa*. Concentrations of up to 100 µM have been detected in the sputa of *P. aeruginosa*-infected CF patients (Wilson *et al.*, 1988), and its production can often be observed visually by colouration of patient sputum samples.

In several infection models, a role for pyocyanin in infection has been demonstrated. For example, pyocyanin production is essential for the success of either acute or chronic lung infection in mice (Lau *et al.*, 2004b). A role for pyocyanin has also been shown in the burned mouse model (Cao *et al.*, 2001), as well as in fast killing of *C. elegans* (Mahajan-Miklos *et al.*, 1999), and the ability of *P. aeruginosa* to kill plants (Rahme *et al.*, 2000) or the fruit fly *Drosophila melanogaster* (Lau *et al.*, 2003).

In addition, there is a substantial body of evidence suggesting that pyocyanin and its derivatives have toxic effects of direct relevance to CF (reviewed by Lau *et al.*, 2004a; Fig. 2). These can be separated into three subcategories: redox, physical and immune response modulation.

Redox effects of pyocyanin

As a zwitterion with both hydrophobic and hydrophilic regions, pyocyanin can easily interact with and penetrate cytoplasmic membranes. Redox cycling, thought to involve the host cell NADH, leads to the production of the reactive oxygen species (ROS) O_2^- and hydrogen peroxide (H_2O_2), with the potential to cause significant oxidative stress on cells (Lau *et al.*, 2004a). Oxidative stress affects calcium homeostasis in a variety of cell types, including human airway epithelial cells, where pyocyanin can lead to elevated concentrations of cytosolic calcium. In addition, pyocyanin interferes with cellular respiration by redirecting electrons down an alternative route, in order to power the redox reactions that lead to the generation of ROS. This leads to a marked depletion of intracellular cAMP and ATP levels. ATP-driven conformational changes control the CFTR chloride channel gates thus controlling chloride ion transport (Gadsby *et al.*, 2006). Therefore, despite the fact that for some CF patients at least, there would be a small number of functional CFTR channels on cell surfaces, any activity could

be further reduced by pyocyanin-mediated ATP depletion. In addition, it has been shown that physiological concentrations of pyocyanin can inactivate airway epithelial vacuolar ATPase, resulting in reduced expression and trafficking of CFTR in cultured lung and primary nasal epithelial cells (Kong *et al.*, 2006).

Endothelial prostanoid production is also redox sensitive. Interestingly, significant inhibition of the release of prostaglandin I2 occurs in the presence of very low concentrations of pyocyanin (1–5 µM) (Kamath *et al.*, 1995). V-ATPases are plasma membrane proteins involved in receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, protein processing and vesicular transport. They also have potential roles in pH homeostasis, K^+ secretion and alveolar macrophage activation. Pyocyanin can inactivate human V-ATPases via the production of ROS.

Human host cells have various mechanisms to counteract the effects of ROS. For example, superoxides are converted to H_2O_2 and water by the enzymes manganese superoxide dismutase and copper–zinc superoxide dismutase. H_2O_2 is removed by the enzyme catalase and cellular thiols, such as glutathione. In cells under oxidative stress, many of these enzymes are upregulated. Pyocyanin can lead to a decrease in the levels of catalase in human alveolar epithelial cells. In addition, pyocyanin directly oxidizes and can deplete levels of glutathione in airway epithelial cells (O'Malley *et al.*, 2004). The exocrine dysfunction associated with CF leads to a systemic deficiency of glutathione and there is evidence that dysfunctional CFTR plays a direct role in this defect (Kogan *et al.*, 2003). The depletion effects caused by pyocyanin could potentially add further to this problem, resulting in reduced defences against oxidative stress, and high levels of oxidative damage in the airways of CF patients.

α1-Protease inhibitor modulates the activity of serine proteases, including human neutrophil elastase, in the lung, protecting tissue from protease-mediated injury. Through redox cycling, pyocyanin can inactivate α1-protease inhibitor. Pyocyanin has also been found to inactivate nitric oxide, which has an array of effects within the human body, exerting major influences over blood flow, blood pressure and immune functions.

Effects of pyocyanin on physical processes

Ciliary clearance of trapped, inhaled particles is one of the first lines of defence against infection of the lungs. Both pyocyanin and its degradation product 1-hydroxyphenazine affect ciliary function (slow and fast action, respectively) leading to slowing of the cilia beat frequency and paralysis of some cilia. In one study, addition of cell free supernatant to airway cilia produced a 58% decrease in cilia beat frequency (Jackowski *et al.*, 1991). This effect would be beneficial to the infectious process in the early stages of infection.

Effects of pyocyanin on the immune response

Neutrophil influx and activation is a major feature of *P. aeruginosa* lung infections. On infection, the neutrophils migrate out of the blood and into tissues towards the site of inflammation by a process of chemotaxis. In the lungs of sheep, pyocyanin causes an inflammatory response characterized by an influx of neutrophils (Lauredo *et al.*, 1998). At concentrations 20-fold lower than the levels found in the sputum of some CF patients, pyocyanin increases the release from human airway epithelia of IL-8, a major chemoattractant for neutrophils (Denning *et al.*, 1998). RANTES [chemokine ligand (CCL)-5] is a chemokine, expressed 3–5 days following T cell activation, that attracts T-cell monocytes and eosinophils to sites of inflammation. This cell influx helps to resolve inflammation whereas any delay can prolong the inflammatory state. Treatment with pyocyanin decreases the level of RANTES released from epithelial cells (Denning *et al.*, 1998).

Neutrophil apoptosis is an important part of normal downregulation of inflammatory reactions. It has been shown that pyocyanin, at clinically relevant concentrations, accelerates neutrophil apoptosis (Allen *et al.*, 2005). This apoptosis is achieved through the generation of ROS and a decrease in intracellular cAMP. More recent work has demonstrated that pyocyanin acts by subverting the lysosomal pathway (Prince *et al.*, 2008) and impairs macrophage engulfment of apoptotic cells (Bianchi *et al.*, 2008).

Pyocyanin inhibits both the production of IL-2 and the expression of IL-2 receptors. IL-2 enhances the production of other lymphokines and is also needed for the proliferation of cytotoxic T cells. These effects combined could mean that not only does pyocyanin prevent the development of an effective T cell response against *P. aeruginosa*, it could also prevent (through the inhibition of cytokine production) activation of monocytes and macrophages. These features could aid in evasion of the immune system and the establishment of chronic infection.

Role of pyocyanin in competition with other microorganisms

Pyocyanin has antibiotic activity against other bacteria and fungi. This activity may allow *P. aeruginosa* an advantage over competing bacteria occupying the same niche. However, at least in some cases, the antibiotic activity of pyocyanin requires an aerobic environment. It has been suggested that the CF lung environment is largely anaerobic due to factors such as the presence of CF airway epithelia that consume two to three times more oxygen than normal airway epithelia, and the high numbers of neutrophils which undergo respiratory burst, further depleting available

oxygen. In this environment, pyocyanin would be much less effective against some competing organisms. Pyocyanin may help *P. aeruginosa* to maintain redox balance by acting as an alternative electron acceptor, especially in low oxygen environments, contributing to the pathogen's adaptability in the CF environment (Price-Whelan *et al.*, 2007).

Other QS-regulated exoproducts in CF

Elastase, encoded by the *lasB* gene, degrades elastin and other matrix proteins of human lung leading to tissue damage and destruction of lung structure. *Pseudomonas aeruginosa* elastase is a potent inflammatory factor in a mouse model of diffuse panbronchiolitis (Yanagihara *et al.*, 2003), and in a rat air pouch inflammation model (Kon *et al.*, 1999). Not only does *P. aeruginosa* elastase degrade surfactant proteins A and D, but degradation fragments are detectable in bronchialveolar lavage samples from lung transplant patients with CF (Mariencheck *et al.*, 2003).

LasA, encoded by the *lasA* gene, is a zinc metalloendopeptidase which has a strong staphylolytic activity and a weaker elastolytic action. Interestingly, LasA increases the elastolytic activity of elastase by nicking the substrate, elastin, making it more susceptible to degradation. The anti-*Stapylococcus* activity of this protein may give *P. aeruginosa* a competitive advantage in the CF niche. LasA also enhances the shedding of the host cell surface molecule syndecan-1, one of a family of cell surface heparin sulphate proteoglycans which have the ability to bind and modulate ligands such as extracellular matrix components, cytokines, chemokines and proteases (Park *et al.*, 2000). The benefits of this process are unclear but it has been suggested that altering target cell surface morphology could disrupt the epithelial barrier leading to enhanced colonization and also that the cleaved soluble syndecan-1 could bind and neutralize proinflammatory mediators such as cytokines and chemokines. These processes could promote bacterial cell survival in the host animal environment (Park *et al.*, 2000).

A study on the effects of alkaline protease on A549 pulmonary epithelial cells suggested that alkaline protease inhibits tumour necrosis factor-α-induced RANTES gene expression and secretion in a concentration-dependent manner (Krunkosky *et al.*, 2005). Another study on airway epithelial cells reported that elastase or alkaline protease degraded human RANTES, monocyte chemotactic protein-1 and epithelial neutrophil-activating protein-78 (Leidal *et al.*, 2003).

Rhamnolipids, encoded by the *rhl* genes, are heat-stable hemolytic glycolipids with detergent-like activity that can be detected in the sputum of CF patients (Kownatzki *et al.*, 1987). Rhamnolipids have cytolytic activity against monocyte-derived macrophages (Fig. 1). More recently, following on from a study demonstrating that *P. aeruginosa* in biofilms

exhibits a QS-regulated tolerance to polymorphonuclear leukocytes (PMNs), Jensen *et al.* (2007) demonstrated that the necrotic effect of culture supernatants from QS-active bacteria against PMNs was caused by rhamnolipids.

Pseudomonas aeruginosa synthesis of hydrogen cyanide (HCN) is regulated by the QS system (Fig. 1). A recent study demonstrated that HCN can be detected in the sputum of *P. aeruginosa*-infected CF patients, and that detection of HCN in sputum was associated with poorer lung function (Ryall *et al.*, 2008). This provides direct evidence for a link between QS-regulated activity during chronic infection and patient morbidity.

Signal molecules interfere with host cell processes

QS signalling molecules, HSLs, can be detected directly in the sputa of CF patients (Singh *et al.*, 2000; Middleton *et al.*, 2002) and sputum extracts can be used to enhance the QS-regulated activities of *P. aeruginosa* (Duan & Surette, 2007). There is evidence that HSLs can enter and function within mammalian cells, and that they may be capable of immune modulation (Shiner *et al.*, 2005). Recently, it was reported that treatment with C12-oxo-HSL can lower the concentration of putrescine in human cells, potentially arresting them in the mitotic phase (Kristiansen *et al.*, 2008).

Does adaptation of *P. aeruginosa* to the CF lung lead to loss of QS?

It has been known for some time that variations in *P. aeruginosa* phenotypes such as morphology, motility and auxotrophy are common in CF infections. These variations, along with changes in antimicrobial susceptibility, are often due to simple mutations. A number of studies have highlighted the accumulation of mutations leading to loss of virulence (Schaber *et al.*, 2004; Smith *et al.*, 2006). In particular, it has been proposed that *lasR* mutants, defective in QS, occur as an adaptation in chronic infections (D'Argenio *et al.*, 2007), and that the QS system may have a negative impact on the organism's long-term fitness (Heurlier *et al.*, 2006).

These ideas have gone a step further, questioning whether QS can be important in pathogenicity if *lasR* mutants are so readily isolated. Cabrol *et al.* (2003) reported that only 50% of clinical isolates had *lasR*-dependent *lasAB* expression, and concluded that this was an indication that QS may not play a significant role in pathogenesis. A similar study concluded that the presence of *lasR* mutants was an indication that QS was not an important factor in the ability of naturally occurring QS-deficient strains to cause infection (Schaber *et al.*, 2004). However, in chronic CF infections even single-strain *P. aeruginosa* populations are communities comprised of mixed phenotypes and genotypes. The presence of *lasR*

mutants may merely indicate that the population has diversified with respect to the QS phenotype. Indeed, it has been suggested that the QS plays a pivotal role in the initial stages of infection, but that the accumulation of *lasR* mutants thereafter may be an indication that the role for QS in pathogenicity diminishes over time (Heurlier *et al.*, 2006). Recently, the cooperative behaviour of mixed populations of bacteria has been studied using populations including both QS wild-type and *lasR* mutants. These studies have introduced the concept of cheating bacteria (the QS mutants) that exploit the functional QS systems of other members of the population (Diggle *et al.*, 2007; Sandoz *et al.*, 2007). However, in order for the social cheats to be maintained, they must not overwhelm the population. Hence, a population balance is maintained between the QS-inactive (cheats) and the QS-active members of the population. Although these studies are based on laboratory cultures carried out under conditions where QS activity is required, it may be that the real chronic infection situation in the CF lung also ensures that, although *P. aeruginosa lasR* mutants accumulate, the QS-active members of the population are still maintained for the benefit of all.

The Liverpool epidemic strain has an unusual QS phenotype

The transmissible Liverpool Epidemic Strain of *P. aeruginosa* is the most prevalent clone amongst CF isolates in the United Kingdom and is associated with increased morbidity. Many isolates of the Liverpool Epidemic Strain, including isolates associated with the transmission to non-CF parents, exhibit an unusual QS phenotype, termed hypervirulence (Salunkhe *et al.*, 2005; Fothergill *et al.*, 2007). The hypervirulence phenotype is characterized by a dysfunctional QS system, leading to production of QS-regulated factors early in the growth phase and to much higher levels overall (Salunkhe *et al.*, 2005; Fothergill *et al.*, 2007). Thus, hypervirulent isolates produce high levels of pyocyanin and other QS-regulated exoproducts, and also exhibit greater killing activity against *Drosophila* and *C. elegans* (Salunkhe *et al.*, 2005; Fig 3). To our knowledge, this unusual phenotype has not been identified in other CF or non-CF isolates of *P. aeruginosa*. Hence, it seems feasible that it might play a role in the success of this epidemic clone, and the greater morbidity associated with it.

The accumulation of *lasR* mutants could be taken as evidence that QS-negative bacteria out-compete QS-competent bacteria in the CF environment. Given that the QS system may be detrimental to the fitness of *P. aeruginosa* (Heurlier *et al.*, 2006), then the unusual hypervirulence phenotype of the Liverpool Epidemic Strain might be expected to amplify any negative impact. However, we found that the hypervirulence phenotype can persist among
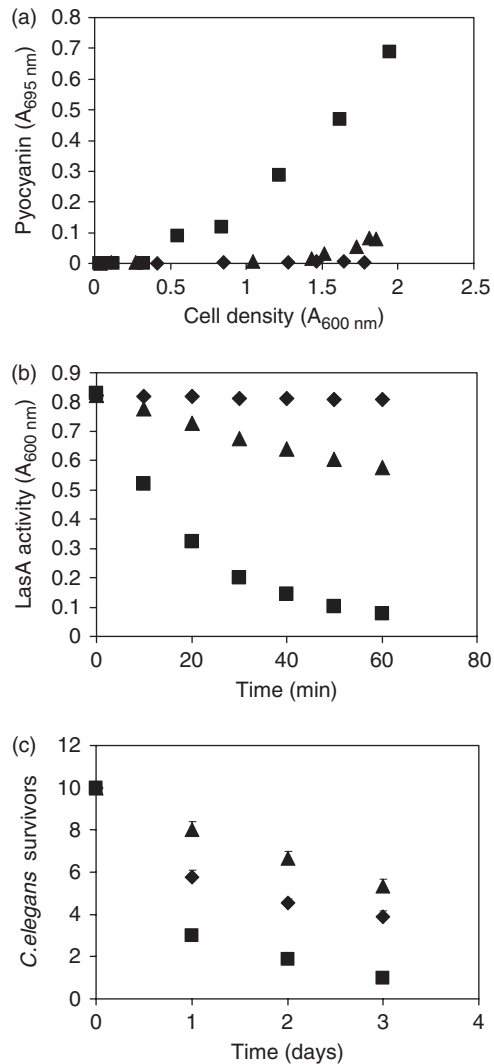
Fig. 3. The novel hypervirulence phenotype of the Liverpool Epidemic Strain. The figure shows example phenotypes exhibited by a hypervirulent isolate (■) in comparison with a *lasR* mutant (♦) and a control strain, PA01 (▲). (a) Pyocyanin production in culture supernatants. (b) LasA activity, measured by the reduction in $A_{600\,nm}$ of a *Staphylococcus aureus* cell suspension upon treatment with *Pseudomonas aeruginosa* culture supernatants. (c) *Caenorhabditis elegans* 'slow killing' assays using groups of 10 nematodes and nine replicates.

the bacterial population infecting CF patients for up to 7 years (Fothergill *et al.*, 2007). It must be assumed, given the size of the QS regulon, that the hypervirulence phenotype represents a considerable burden on those members of the Liverpool Epidemic Strain population maintaining it. The fact that the phenotype is retained suggests either that it continues to provide a selective advantage for individual members of the population, compensating for any negative burden, or that the CF *P. aeruginosa* population is cooperative in nature, with non-QS bacteria exploiting the activity of the hypervirulence bacteria, which nevertheless provide an essential service for the population as a whole and are therefore maintained. Certainly, the maintenance of this phenotype amongst the population argues in favour of an ongoing role for QS during chronic infection in CF.

Interspecies communication

There is evidence that QS-mediated cross-communication can occur between *P. aeruginosa* and the *Burkholderia cepacia* complex within mixed biofilms (Riedel *et al.*, 2001). Interactions between the QS systems of *P. aeruginosa* and fungi have also been reported (McAlester *et al.*, 2008). However, experiments using the sputa of CF patients suggest other intriguing possibilities for interspecies communication. The presence of avirulent oropharyngeal flora isolated from sputum samples of CF patients can enhance the lung damage caused by *P. aeruginosa* in a rat lung infection model (Duan *et al.*, 2003). Transcriptome profiling suggested that some of the *P. aeruginosa* genes affected were QS-related.

QS as a therapeutic target

There has been a lot of interest in the prospect of targeting the QS system for therapeutic purposes (Martin *et al.*, 2008). The main strategies are outlined in Table 1. The fact that macrolides have been identified as QS inhibitors (Tateda *et al.*, 2007) is of great interest. Clinical trials have demonstrated that long-term treatment with azithromycin can have significant benefits for lung function in CF patients (reviewed by Tateda *et al.*, 2007). Azithromycin normally acts by inhibiting protein synthesis, but *P. aeruginosa* is

Table 1. Therapeutic strategies against the QS system

Agent	Comments	References
Macrolides	Inhibition of QS-regulated factors; inhibition of alginate production and biofilm formation; several azithromycin trials in CF patients showed beneficial effects for lung function	Tateda *et al.* (2007)
Synthetic analogues	Synthetic autoinducer analogues/ligands; inhibition of QS-regulated factors and biofilm formation	Geske *et al.* (2007)
Garlic	QS is inhibited by garlic and renders *P. aeruginosa* more sensitive to tobramycin and host defences	Bjarnsholt *et al.* (2005)
QS vaccine	Specific antibody to 3-oxo-C12-HSL plays a protective role in acute *P. aeruginosa* infection	Miyairi *et al.* (2006)
Plant extracts	Inhibition of LasA protease, LasB elastase, and biofilm formation by several different plant extracts	Adonizio *et al.* (2008)

generally considered to be resistant to this mode of action. Azithromycin can lead to inhibition of protein synthesis by 80% leading to reduced expression of various exoproducts, including pyocyanin (Wagner *et al.*, 2005). Furthermore, in a CF chronic infection model, azithromycin treatment suppresses QS-regulated factors leading to reduced lung pathology and biofilm formation (Hoffmann *et al.*, 2007).

Conclusion

Much of the published work on QS in relation to *P. aeruginosa* infections in CF supports a role for QS early in the infection process, but loss of the phenotype over time indicates a diminishing role thereafter. However, the detection of continuing QS activity directly in CF patient sputa, coupled with recent developments in understanding the cooperative behaviour of mixed phenotype bacterial populations, suggest an ongoing role for QS. While we still lack a detailed understanding of how QS systems really operate during the infection process, strategies for novel therapeutics aimed at attenuation of QS show great promise. It is clear that bacterial population behaviour in CF is complex, and much more research is required before we will understand the role of QS.

Acknowledgement

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