



Is Quorum Sensing Interference a Viable Alternative to Treat *Pseudomonas aeruginosa* Infections?

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Quorum sensing (QS) coordinates the expression of multiple virulence factors in *Pseudomonas aeruginosa*; hence its inhibition has been postulated as a new alternative to treat its infections. In particular, QS interference approaches claim that they attenuate bacterial virulence without directly decreasing bacterial growth and suggest that *in vivo* the immune system would control the infections. Moreover, since *in vitro* experiments performed in rich medium demonstrate that interfering with QS decreases the production of virulence factors without affecting bacterial growth it was assumed that *in vivo* therapies will minimize the selection of resistant strains. Therefore, the underlying assumptions toward an effective implementation of a successful Quorum sensing interference (QSI) therapy for treating *P. aeruginosa* infections are that (i) QS only exerts important effects in the regulation of virulence genes but it does not affect metabolic processes linked to growth, (ii) the expression of virulence factors is only positively regulated by QS, (iii) inhibition of virulence factors *in vivo* do not affect bacterial growth, (iv) the immune system of the infected patients will be able to get rid of the infections, and (v) the therapy will be effective in the strains that are actively producing the infections. Nevertheless, for QSI in *P. aeruginosa*, substantial experimental evidence against the validity of most of these assumptions has accumulated during the past years, suggesting that a far better understanding of its virulence and its behavior during infections is needed in order to design truly solid QSI therapeutic alternatives to combat this remarkable pathogen.

Keywords: *Pseudomonas* infections, quorum sensing (QS), quorum quenching, resistance, clinical isolates

INTRODUCTION

Pseudomonas aeruginosa is a remarkable opportunistic pathogen that infects patients that are immunocompromised, have severe burns, cancer, or AIDS, are intubated and with prosthetic devices, and also those suffering from chronic affections like cystic fibrosis (CF). This bacterium is a major health problem worldwide being responsible of 10% of nosocomial infections (Antunes et al., 2010; Castillo-Juárez et al., 2015), since it is intrinsically resistant to several antimicrobials (Poole, 2011) and able to develop resistance against new ones, has a high biofilm production and produces an arsenal of virulence factors. One of the main mechanisms that controls the production of its virulence factors is quorum sensing (QS) which coordinates the expression of such factors

once bacteria have reached a high population density, thus maximizing their chances to overcome the host defenses and establish the infection.

QS systems are common in bacterial pathogens; among Gram negatives, in addition to *P. aeruginosa*, *Acinetobacter baumannii* (Bhargava et al., 2010), *Escherichia coli* (Sperandio et al., 2002) *Salmonella* strains (Choi et al., 2007), and *Vibrio* strains (Zhu et al., 2002; Yang and Defoirdt, 2015) use them to coordinate their virulence. Hence, QS interference (QSI) or quorum quenching (QQ) is a strategy proposed to inhibit virulence as an alternative to treat the infections of several important bacterial pathogens (Castillo-Juárez et al., 2015).

In this opinion piece I focus in *P. aeruginosa*, one of the most studied organisms regarding QS and QSI, nevertheless what is exposed here may be also applicable to other bacterial pathogens.

P. aeruginosa is one of the more complex known bacterial pathogens; it possesses an ample genome and a high percentage of its genes are devoted to gene regulation (Stover et al., 2000). Regarding QS systems, it has a hierarchical architecture governed by the LasRI module which produces and senses N-3-oxo-dodecanoyl-L-Homoserine lactone and activates the expression of multiple virulence genes such as those producing elastase A and B, pyocyanin, alkaline and protease, and activating a second homoserine lactone (HSL) QS module known as RhlRI, which produces and senses N-butyryl-L-Homoserine lactone. RhlR bound to its autoinducer also activates directly some virulence genes like those encoding rhamnolipids and pyocyanin (Smith and Iglewski, 2003; Jimenez et al., 2012). In addition, *P. aeruginosa* also has a quinolone dependent system known as PQS, which is positively regulated by LasR and negatively by RhlR, forming a complex and intricate network (Jimenez et al., 2012; Lee and Zhang, 2015). In addition to the QS intrinsic components, several regulators such as GacA/GacS, QscR, Vfr, RpoN, and RpoS, influence the expression of QS dependent factors (Lee and Zhang, 2015).

The complex relationship between QS and virulence in *P. aeruginosa* had been recently evidenced by the fact that *lasR* deficient mutants (including clinical strains) growing at slow rates or in the stationary phase, overproduce pyocyanin due a lack of repression of the phenazine genes by RsaL, a negative transcriptional regulator positively controlled by LasR (Cabeen, 2014). Moreover, in *lasR* mutants, the activity of the *rhl* system is only delayed but not abolished, allowing significant production of pyocyanin, rhamnolipids and N-butyryl-L-Homoserine lactone (which are RhlR dependent) and even the production of the LasR dependent QS signals N-3-oxo-dodecanoyl-L-Homoserine lactone and PQS (Dekimpe and Deziel, 2009). These findings suggest that the inhibition of a particular component of the QS network, even the pivotal factor LasR, may be counteracted by the activation of alternative components of the network. Indeed, some recent studies have identified novel QS inhibitors for which RhlR and not LasR as the relevant *in vivo* target (O'Loughlin et al., 2013; Welsh et al., 2015).

In addition, recently it was discovered that an environmental strain (148 isolated from dolphin gastric juice) produces significant amounts of the QS-controlled virulence factors rhamnolipids and pyocyanin. Critically, this strain is virulent

to mice, even without having a *lasR* gene and without producing N-3-oxo-dodecanoyl-L-Homoserine lactone. Hence, in this strain, the control of QS dependent virulence factors must be exerted by other regulators (Grosso-Becerra et al., 2014).

QS Interference could be achieved by the attenuation of the QS communication systems via: (i) the disruption of the QS receptors, (ii) the degradation of the autoinducers signals or (iii) the inhibition of the signal synthesis. Accordingly, several QS inhibitors or quorum quenchers (QQ) suitable for *P. aeruginosa* have been developed, under the assumptions that (i) the expression of virulence factors is only positively regulated by QS, (ii) the QQ will only exert significant effects in the regulation of virulence genes but not in metabolic processes linked to growth, hence avoiding or at least decreasing the generation of selective pressure that leads to resistance, (iii) the immune system of the infected patients will be able to get rid of the infections, and (iv) the therapy will be effective against the strains that are actively producing the infections. Nevertheless, substantial evidence against the validity of those assumptions has accumulated over the years.

QUORUM SENSING INFLUENCES METABOLIC PROCESSES AND BACTERIAL GROWTH

Although the role of QS in *P. aeruginosa* virulence has been extensively studied, whether it influences global metabolism and cell growth is less explored. One possible reason for this bias is that at the transcriptomic level, few significant changes in metabolically related genes are found when QS proficient and deficient strains are compared or when QS inhibitors are administered to QS proficient strains (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). Nevertheless, recently Davenport and coworkers found that the metabolome of a *lasI rhlI* double mutant (unable to produce HSL QS autoinducers) and that of its wild-type progenitor have a notorious divergence once the wild-type strain produced the highest levels of autoinducers; remarkably, around one third of all the metabolites identified changed (Davenport et al., 2015). This phenomenon is understandable since the wild-type strain devotes many of its resources to the production of costly virulence factors such as exoproteases, phenazines, and exopolysaccharides, while, in contrast, the QS mutants uses the same resources for cell division. In agreement, the QS deficient mutants achieve higher growth yields than QS wild-type strains (Diggle et al., 2007).

Although the possible implications of the metabolic divergence in QS mutants on bacterial physiology and QS interference therapies is still unexplored, Davenport and coworkers demonstrated that important metabolic changes in the membrane metabolism of the wild-type strain are driven by QS upon entering the stationary phase. These modifications include increased fatty acid saturation, chain length, and cyclopropanation, which in turn, promote the generation of robust cell membranes. In contrast, the membranes of QS mutants do not have these modifications and are therefore more susceptible to stress.

In addition to these metabolic changes, several works have demonstrated that in *P. aeruginosa* and other bacteria, QS enhances the stress response (Bjarnsholt et al., 2005; Bhargava et al., 2014; de Oca-Mejía et al., 2015; García-Contreras et al., 2015a); for example, by upregulating antioxidant enzymes such as catalase, superoxide dismutase (Hassett et al., 1999) and NADPH-generating enzymes (García-Contreras et al., 2015a). This is important since during an infection, the immune system attacks bacteria by releasing reactive oxygen species, and at least *in vitro* oxidative stress is able to select functional QS systems as well as QS interference resistant mutants (García-Contreras et al., 2015a).

Moreover, growth of *P. aeruginosa* can be strongly dependent on QS either if adenosine or protein is used as sole carbon sources, since *nuh*, the gene encoding the nucleoside hydrolase, is under tight LasR control (Heurlier et al., 2005) and since the expression of exoproteases is under QS control (Diggle et al., 2007). During infections, adenosine utilization (Patel et al., 2007; Sheng et al., 2012) as well as the degradation of host proteins (Wretling and Pavlovskis, 1983; Laarman et al., 2013) are important for bacterial virulence and survival.

All the above summarized facts demonstrate that QS is an important regulator of bacterial metabolism and physiology and suggest that QS interference will have the direct effect of decreasing bacterial growth and viability *in vivo*. In agreement, several *in vivo* infection studies have shown that when a QS interference therapy is able to increase animal survival (and decrease damage to the host), there is a significant decrease in bacterial counts in the infection sites (Wu et al., 2004; Christensen et al., 2007; Defoirdt et al., 2010; Jakobsen et al., 2011). The decrease in bacterial viability due QS interference may be an important potential source of *in vivo* selective pressure for the selection of bacterial resistance (García-Contreras et al., 2016).

POTENTIAL INCREASE IN VIRULENCE UPON QUORUM SENSING INTERFERENCE

Although the production of multiple virulence factors in *P. aeruginosa* is positively regulated by QS, the virulence phenotype displayed by this organism is a complex combinatorial phenomenon that cannot be easily predicted with the identification of the presence or absence of a set of specific genes (Lee et al., 2006; Grosso-Becerra et al., 2014). In agreement, several studies had shown that clinical strains display a wide variety levels of virulence (Fenner et al., 2006; Lee et al., 2006; Garcia-Contreras et al., 2015b) and that environmental strains often conserve high virulence (Grosso-Becerra et al., 2014).

An overlooked fact about the role of QS in *P. aeruginosa* virulence was discovered in 2005 by Bleves and coworkers, who that found that in contrast to several other virulence traits, the expression of the type III secretion system (TTSS) in *P. aeruginosa* is negatively regulated by QS, specifically by RhlR and PqsR (Bleves et al., 2005; Kong et al., 2009). An important factor in such negative regulation of virulence genes by QS is that

it requires low calcium levels, and hence several transcriptomics studies done in culture medium such as LB with relative high calcium levels fail to show this relationship. Since the TTSS is an important determinant of virulence in several animal infection models including pneumonia, peritonitis, bacteremia, burn infections, and keratitis (Hauser, 2009), the possibility that QS interfering therapies may be activating this system and therefore promoting virulence should not be ignored. Moreover, *P. aeruginosa* has five of the six known types of secretion systems present in Gram negative bacteria (all but type IV) and some of these systems, like type VI, are present in several copies in its genome (Bleves et al., 2010). The QS influence on the expression of these secretion systems is as of yet unknown, with the exceptions of the TTSS and the second type VI secretion system that are negatively regulated by QS (Sana et al., 2012).

More striking is the fact that QS interference using azithromycin selects the wild-type virulent phenotype against the less virulent *lasR* mutants in intubated patients colonized by *P. aeruginosa* (Kohler et al., 2010). This surprising fact is understandable since *lasR* mutants often appear and are selected in infections since they act as phenotypic cheaters that utilize the public goods such as exoproteases and siderophores produced by the cooperative wild-type individuals (Diggle et al., 2007; Sandoz et al., 2007). Hence, inhibiting QS removes the advantage of the *lasR* mutants and thereby selects the wild-type, thus a detrimental effect of QS interference in the long run could be to increase the prevalence of virulent genotypes in the nosocomial environment (Kohler et al., 2010).

Another key aspect of current QS inhibitors like furanones is that, depending on their concentration, they can activate rather than inhibit QS (Martinelli et al., 2004). Furthermore, related QS inhibitors such as synthetic HSLs can activate rather than inhibit some virulence factors (Welsh et al., 2015).

LIMITATIONS OF THE CURRENT ANIMAL INFECTION MODELS FOR THE STUDY OF QUORUM SENSING INTERFERENCE

To date, several animal infection models have demonstrated that QS deficient mutants are much less virulent than their QS proficient parental strains; accordingly, QS interference promotes an increase in host survival and a decrease in damage and bacterial counts (Castillo-Juárez et al., 2015). The infection models used include arthropods like *Galleria mellonella*, nematodes such as *Caenorhabditis elegans*, fruit fly, and zebrafish which are valuable and informative but that do not accurately reflect human physiology. Moreover, when mice are used as a model, immune competent individuals are evaluated; hence, the fact that *P. aeruginosa* is a strict opportunistic pathogen that does not attack individuals with competent immune systems is overlooked, and so it is not clear if the immune systems of immunosuppressed patients will be able to clear the bacteria from infections upon QS interference treatments.

In addition to attacking immunosuppressed individuals, *P. aeruginosa* is the major cause of death of CF patients; accordingly, there are several mice models that incorporate

different mutations in the CF transmembrane conductance regulator (CTFR) protein and that are able to mimic some of the characteristics of the disease in humans. However, CF is a very complex disease and there are more than one thousand reported mutations in the *cfr* gene associated with the disease; hence, developing a murine model that closely resembles the human disease is challenging (Guilbault et al., 2007).

Most of the current models are suitable to study acute infections and only a few like the one developed in 2005 by Hoffmann and coworkers are optimized to simulate chronic CF infections (Hoffmann et al., 2005). Importantly, this model has been used to demonstrate that QS interference with azithromycin inhibits alginate production of the mucoid NH57388A strain *in vivo*, attenuating the damage produced to the host, but unfortunately it was not able to significantly increase mouse survival (Hoffmann et al., 2007).

Clinical studies have demonstrated that AZM treatment improves lung function in CF patients (Saiman et al., 2003); however, besides the QSI effect, azithromycin has bactericidal and anti-inflammatory effects; hence, the improvement of both mice and patients is likely due a combination of these effects rather than an exclusive consequence of the QSI properties of azithromycin. Therefore, testing of the effect of more specific QS interference molecules in these kind of models is needed in order to elucidate the potential of these therapies for CF patients.

Another kind of mouse model that more closely resembles the situation observed in humans is the thermally-induced injury model, which consists of producing a burn of second or third degree on the dorsal side of the mouse using hot water and subsequent inoculation of the burn. The utilization of this model has confirmed that QS-deficient mutants such as *lasR*, *lasI*, *rhlI*, *lasI rhlI*, and *pqsA* (Rumbaugh et al., 1999a,b; Lesic et al., 2007), have less virulence than the parental strains. Moreover, it has been used to test the effect of the halogenated anthranilic acid analogs 6FABA, 6CABA, and 4CABA which are inhibitors of the synthesis of quinolone QS signals. These compounds, at the doses administrated, decrease mouse mortality significantly. Nevertheless, they were tested at a single dose (Lesic et al., 2007), so it is unknown if they exert dose response effects. This is not trivial since as mentioned before, QS inhibitors may act as QS activators depending on the used doses (Martinelli et al., 2004).

INSENSIBILITY OF SOME CLINICAL STRAINS TO CURRENT QUORUM QUENCHERS

Before 2010 it was assumed that QS interference will be impervious or at least less susceptible to promote bacterial resistance than conventional antibiotic therapies (Defoirdt et al., 2010). In 2011, it was demonstrated that *P. aeruginosa* acquires resistance easily against the canonical HSL-dependant quorum quencher furanone C-30, by activating the multidrug efflux pump MexAB-OprM (Maeda et al., 2012). The activation of this pump is mediated by mutations disrupting the transcriptional repressors MexR and NalC, and such mutations are common in

clinical isolates, presumably since they are selected by intense antibiotic treatments (Tomas et al., 2010). As expected, these clinical isolates are resistant against C-30 (Maeda et al., 2012).

Recent studies have demonstrated that C-30 resistance is common in multidrug resistant strains. Importantly, resistance against other QQ compounds like 5-fluorouracil is also present in some clinical isolates and some antibiotic sensitive strains are also resistant to C-30 by a decrease in the compound uptake. More strikingly, some of these strains produce higher amounts of the virulence factors in the presence of the furanone than in basal conditions (García-Contreras et al., 2013a,b; Garcia-Contreras et al., 2015b).

This suggests that if eventually QSI therapies are implemented in the clinic, they likely would not be effective against all the strains present in infections (García-Contreras et al., 2013a; Kalia et al., 2013), and that crucially they may fail to inhibit those strains with active antibiotic efflux pumps. Indeed, it was recently proposed that bacteria resistant to several QS inhibitors may be selected by QSI treatments (Koul et al., 2016).

PERSPECTIVES

Since recent evidence has demonstrated that QS is linked to basal metabolism and growth, it will be important to evaluate QS interference and its effect in bacteria growing in medium that more closely simulates the composition of the environment during infection (Palmer et al., 2007; Garcia-Contreras et al., 2015b), with attention on key metabolites that influence virulence and QS such as iron (Sokol and Woods, 1984; Mittal et al., 2008; Hazan et al., 2010), calcium (Sarkisova et al., 2005), phosphate (Zaborin et al., 2009), and adenosine (Patel et al., 2007; Sheng et al., 2012). Further, metabolomic and proteomic studies may shed light about the role of QS in bacterial physiology under *in vivo*-like conditions. In addition, the utilization of new technologies like microfluidics such as cell culture chips (organ-on-a-chip) would enable the interrogation of the roles of signaling, social cheating, mass transfer, and spatial organization in well-defined geometries, which is virtually impossible to assess at this moment using animal models.

Importantly, additional research about the ways *P. aeruginosa* may achieve resistance against QQ is needed in order to generate effective combination therapies less prone to the selection of resistance. One possibility is to exploit the fact that QS interference renders *P. aeruginosa* more sensitive to stress, including antibiotics and the effects of the immune system; hence, it is attractive to test if the combination of QS interference, antibiotic therapies and immunotherapy to improve the outcome of the therapies.

Another area of research that should be encouraged is the role of QS independent virulence factors and those negatively regulated by QS such as TTSS in infections and the effect of QS interference on these virulence determinants. Also, study of QS networks and the-virulence of environmental and clinical strains is needed since current evidence indicates these are highly variable (Grosso-Becerra et al., 2014; Garcia-Contreras et al.,

2015b) and that a specific QQ compound will not always be effective against all clinical strains. Also, more effort should be devoted to the study of the implementation of these therapies, since most of them are able to attenuate infections when the QQ compound is administered shortly before the inoculation (having possible prophylactic effects), but the value of such therapies as a possible cure for an established infection should be better addressed.

Taking the available evidence together, it seems QS interference may be a valuable tool to combat *P. aeruginosa* infections; however, using QS inhibition as the sole therapy may not be an efficient strategy due several potential drawbacks. Hence, before QSI applications are used in the clinic, it is advisable to improve our current understanding of *P. aeruginosa* virulence with an emphasis on clinical strains and also on further defining the roles of QS in the physiology of this remarkable complex pathogen.

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AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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