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Secretion of *Pseudomonas aeruginosa* Type III Cytotoxins is Dependent on *Pseudomonas* Quinolone Signal Concentration

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that can, like other bacterial species, exist in antimicrobial resistant sessile biofilms and as free-swimming, planktonic cells. Specific virulence factors are typically associated with each lifestyle and several two-component response regulators have been shown to reciprocally regulate transition between biofilm-associated chronic, and free-swimming acute infections. Quorum sensing (QS) signal molecules belonging to the *las* and *rhl* systems are known to regulate virulence gene expression by *P. aeruginosa*. However the impact of a recently described family of novel quorum sensing signals produced by the *Pseudomonas* Quinolone Signal (PQS) biosynthetic pathway, on the transition between these modes of infection is less clear. Using clonal isolates from a patient developing ventilator-associated pneumonia, we demonstrated that clinical observations were mirrored by an *in vitro* temporal shift in isolate phenotype from a non-secreting, to a Type III cytotoxin secreting (TTSS) phenotype and further, that this phenotypic change was PQS-dependent. While intracellular type III cytotoxin levels were unaffected by PQS concentration, cytotoxin secretion was dependent on this signal molecule. Elevated PQS concentrations were associated with inhibition of cytotoxin secretion coincident with expression of virulence factors such as elastase and pyoverdine. In contrast, low concentrations or the inability to biosynthesize PQS resulted in a reversal of this phenotype. These data suggest that expression of specific *P. aeruginosa* virulence factors appears to be reciprocally regulated and that an additional level of PQS-dependent posttranslational control, specifically governing type III cytotoxin secretion, exists in this species.

Keywords

Pseudomonas quinolone signal; Type III secretion; *Pseudomonas aeruginosa*; ventilator-associated pneumonia; endotracheal aspirate

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Introduction

Pseudomonas aeruginosa is a ubiquitous environmental gram negative bacterium that can cause a variety of acute and chronic infections (1). This pathogenic plasticity is attributed to the large, complex genome encoding a vast arsenal of virulence factors and regulatory switches which permit *P. aeruginosa* to thrive in a multitude of niches and rapidly adapt to fluctuations in environmental conditions (46). Like many other bacterial species, *P. aeruginosa* can form biofilms, communities of bacterial cells encased in an exopolysaccharide matrix that protects the cells within from antimicrobial activity and host immune responses (27,33,39). *In vitro*, biofilms have been associated with production of specific virulence factors such as elastase and pyocyanin (14) which are regulated by quorum sensing molecules, diffusible signaling compounds produced by bacterial species (27,33,39). Chronic infections, including pulmonary disease of cystic fibrosis patients, have been associated with *P. aeruginosa* biofilms (5,30, 44), elevated elastase secretion (17) and high concentrations of quorum sensing signals in respiratory samples (43).

Outside of the biofilm, bacteria may also exist as free-swimming or planktonic cells. Motile free-swimming *P. aeruginosa* cells are associated with acute infections and secrete a number of cytotoxins through a needle-like complex, the type III secretion system [TTSS;(2,41)]. *Pseudomonas aeruginosa* communities are in constant state of flux with planktonic cells capable of detaching from the biofilm, suggesting that both modes of lifestyle can exist in a given niche in parallel. This is supported by recent findings demonstrating the presence of type III cytotoxins in the flow through from biofilm cultures of *P. aeruginosa* (32). Transition between biofilm and planktonic forms appears to involve reprogramming of a number of physiological aspects, including lipopolysaccharide structure, putatively to facilitate the virulence factors expressed during each mode of lifestyle (2). Transition between *P. aeruginosa* biofilm and free-swimming cells is known to be controlled by a number of two component regulators e.g. the Sad, Lad, Ret and Gac sensor-response systems, which clearly respond to specific environmental signals (25,47). Regulatory interplay between these molecular switches is postulated to fine tune reciprocal expression of a number of genes, including those typically associated with chronic or acute infection (19,47).

Regulation of type III secretion genes has previously been associated with the *las* and *rhl* quorum sensing system (6), a cell-to-cell signaling mechanism that permits rapid response to environmental changes through production of diffusible molecules that, when sensed by their cognate cell surface associated receptor, regulate gene expression. This sophisticated communication system, enables coordinated gene expression in bacterial populations facilitating cooperative behavior by bacterial communities. *P. aeruginosa* coordinates population gene expression primarily through three main quorum sensing systems: the *las* and *rhl* systems which employ acylated homoserine lactone signal molecules (3O-C₁₂-HSL and C₄-HSL, respectively), and the recently described 2-alkyl-4(1-*H*)-quinolone (AHQ) family (3,48) The AHQ's include more than 50 quinolone compounds, two of which, *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) and its immediate precursor, 2-heptyl-4-quinolone (HHQ) have been shown to act as quorum sensing signals (16).

Here we describe the basis for altered virulence profiles in a set of clonal *P. aeruginosa* clinical strains isolated from a single patient developing ventilator-associated pneumonia. In parallel with clinical observations made over a period of 3 weeks, *in vitro* observations suggested that these serial clonal isolates transitioned from a non-secreting to an acute, type III-secreting phenotype. This transition coincided with a shift in the concentration of extracellular secreted organic molecules (including PQS) produced by the strains. The effect of PQS concentration on type III secretion was confirmed by a series of experiments exposing strains to cell-free supernatant from PQS-sufficient or -deficient strains and by inhibiting quinolone production.

These data suggest that *P. aeruginosa* strains have the ability to transition over relatively short periods of time to acutely infectious cells and that type III cytotoxin secretion, a hallmark of acute infection by this species, is regulated by the relatively recently described quinolone signal, PQS. That this phenotype was maintained *in vitro* suggests that it is “hard-wired” into the genetic blueprint of the organism. These observations open up the possibilities for novel therapeutic approaches involving physiological transition of this species towards the more antimicrobial susceptible free-swimming lifestyle by blocking quinolone production in combination with antimicrobial and anti-type III secretion virulence therapies in cases of refractory chronic infection.

Materials and Methods

P. aeruginosa clinical isolates used in this study

P. aeruginosa isolates were obtained from endotracheal aspirates (ETA), collected sequentially from a single patient who was culture-positive for *P. aeruginosa* and subsequently met the criteria for ventilator associated pneumonia (VAP) following three weeks of mechanical ventilation. Five clonal isolates from this patient collected pre- and post-VAP diagnosis were used for this study (Table 1). For all experiments, fresh cultures generated from glycerol stocks of these isolates were used to ensure that the strains maintained their phenotype during the *in vitro* experiments performed.

Pulsed field gel electrophoresis (PFGE) analysis of isolates

P. aeruginosa isolates were cultured overnight in Luria broth at 37°C with shaking. Agarose plugs containing 5×10^8 cells/ml of overnight culture were prepared according to the Bio-Rad PFGE protocol (Bio-rad Laboratories, CA) prior to overnight digestion using 40 units of *SpeI* at 37°C. PFGE was carried out using a Lambda ladder size standard (Bio-Rad Laboratories, CA) and a CHEF-DR III apparatus (Bio-Rad Laboratories, CA). PFGE conditions were as follows: initial switch time 1.0 s, final switch time 25 s, total run time 24 h, included angle 120°C, electric field 6 V/cm, buffer 0.5x TBE, temperature 14°C, and agarose 1% pulsed field certified (Bio-Rad Laboratories, CA).

Pyoverdinin and Elastase assays

Isolates were cultured in LB broth overnight prior to dilution (OD_{600} , 0.1) in fresh medium and reincubation for 24 h at 37 °C with shaking. Spectrophotometric measurements (405 nm) of triplicate cell-free supernatants from each strain were performed to determine pyoverdinin production. Parallel elastase activity was determined in these same cultures using elastin congo red and spectrophotometric measurements at 490 nm, according to methods previously described by Nicas *et al* (36).

Type III secretion assay

Overnight cultures of each strain in MIN-S medium (37) were diluted to an OD_{600} of 0.1 in fresh MIN-S and incubated with shaking for a further 4 h. Cultures were harvested by centrifugation, the cell-free supernatant was removed and concentrated using centricon tubes (MWCO = 10,000; Millipore, MA). The cell pellet was lysed using SDS buffer (2% SDS in Tris-HCl, pH 7.0). Protein concentration for both fractions was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, CA) according to the manufacturer’s instructions. Standardized protein concentrations ($20 \text{ ng } \mu\text{g}^{-1}$) of cell lysate and cell-free supernatant (secreted) fractions from each isolate were electrophoresed on a 12% Criterion SDS polyacrylamide gel (Bio-rad Laboratories, CA), prior to transfer onto PVDF membrane (Amersham, NJ) and immunoblotted using antibodies against ExoU, ExoT as previously described (2).

Cytotoxicity assay using a BEAS2-B lung epithelial cell line

Human immortalized BEAS-2B lung epithelial cells (200 μ l) were seeded at a density of 2.5×10^5 ml⁻¹ in Dulbeccos Modified Eagle Medium supplemented with 10% BCS in 96-well cultures and incubated overnight to reach confluency. Each *P. aeruginosa* isolate was grown overnight in LB medium as described above. Strains were harvested, washed three times in sterile PBS, and finally resuspended in 100 μ l Ringers: PBS solution (2:1). Each well received 5×10^6 bacteria. Samples were taken at 30 min time intervals and cytotoxicity assayed by lactate dehydrogenase release using the Cyto Tox96® kit (Promega, CA) according to the manufacturer's instructions.

Quinolone Signal assays

Thin layer chromatography analysis of organic compound production

Each strain was cultured overnight in LB as described above, prior to dilution to OD₆₀₀ of 0.1 and reincubation for 24 h. Cell-free supernatant was then removed from harvested cultures, using methods as described above. Pooled extracts of 1 volume of acidified ethyl acetate (0.1% glacial acetic acid [v/v]) performed twice, which has been shown to extract PQS and other organic compounds (8), was performed on each cultured supernatant prior to quantification by TLC.

Real-time quantitative PCR for expression of *pqsA*

Each strain was cultured overnight in LB medium as described above, diluted to an OD₆₀₀ of 0.1 and reincubated for 4 h. Total RNA was purified using Trizol (Invitrogen, CA) according to the manufacturer's instructions. After incubation with DNase I (Invitrogen, CA), total RNA was checked for integrity by gel electrophoresis, and absence of DNA by PCR, prior to cDNA synthesis. RNA from each strain was quantified and cDNA generated using 1 μ g total RNA and Omniscript reverse transcriptase (Qiagen, CA). One μ l of this reverse transcriptase reaction was used for each Q-PCR reaction. Q-PCR primers for *pqsA* (*pqsAF*, 5'-GAGTGCCTTCCATCGCCAGG-3' and *pqsAR*, 5'-AGCGAACGCCCGGCAGAAAC-3'), the first gene in the PQS biosynthetic operon, were used to quantify expression in all 5 isolates. mRNA transcript copy number was measured using the QuantiTect SYBR Green PCR Kit (Qiagen, CA) according to the manufacturer's instructions. Reactions were carried out using a Mx3000P Real-Time PCR System (Stratagene, CA) as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 30 s. The data acquisition step was set at 58°C with a final melting-curve analysis to ensure amplification of a single product. Quantification of *pqsA* copy number was determined using a standard curve of known *pqsA* copy number, as described in the manufacturer's instructions (Qiagen, CA).

Exposure of clinical isolates to exogenous PQS-sufficient or deficient supernatant extracts

Large scale (2 $\times$ 200 ml LB) preparations of cell-free quinolone signal extracts were carried out on overnight cultures of clinical strain 1 (PQS-sufficient), the laboratory strain PAO1 (PQS-sufficient), or PAO1 Δ *pqsH* (generously provided by Stephan Heeb and Paul Williams, University of Nottingham, UK), which produces the PQS precursor, 4-hydroxy-2-heptylquinoline (HHQ) and related AHQs, but not PQS (16). Extracts were prepared using 1 volume of acidified ethyl acetate as described above. Extractions were pooled and 40 ml of this pooled solution added to each 50 ml falcon tube prior to drying under compressed air. Each *P. aeruginosa* isolate was cultured overnight in MIN-S and diluted to an OD₆₀₀ of 0.1 in fresh MIN-S. Twenty ml of each diluted culture was placed in a 50 ml Falcon tube (screw caps were replaced with cotton bungs) that contained dried quinolone signal extract (PQS-sufficient or deficient extracts), a set of tubes containing 20 ml of culture but no quinolone extract served as controls. All tubes were incubated for 24 h with shaking at 37°C. Following incubation, 5

ml of culture from each of the quinolone-containing and control tubes was harvested for immunoblot analysis using anti-ExoU antibody to assess cytotoxin secretion profiles as previously described (2).

Exposure of clinical isolates to farnesol

Overnight MIN-S cultures of each strain were diluted to an OD₆₀₀ of 0.1 in fresh MIN-S. To 5 ml of diluted culture, a final concentration of 10 mM farnesol, a compound reported to inhibit PQS (11), was added. Cultures were incubated with shaking for a further 4 h. Following incubation, each culture was harvested for immunoblot analysis with ExoU to assess cytotoxin secretion, as described previously (2).

Results

Clonal isolates from a patient developing VAP exhibit a temporal shift in virulence phenotype

Visual inspection of the 5 sequential clinical samples collected from a single patient who developed VAP demonstrated a notable temporal change in appearance. Initial ETA samples (1 and 2) appeared green, while the latter three (3, 4 and 5) became progressively bloodier (Fig. 1A), suggesting temporal progression of the lower respiratory tract infection from a chronic (pyocyanin-producing) to an acute (type III secretion) pathology. Examination of the *P. aeruginosa* colonies isolated from these ETA's on *Pseudomonas* isolation agar revealed a corresponding change in colony morphology. The dominant morphology (~ 95% of all colonies) of colonies cultured from these first two ETA's (strain 1 and 2) was glossy and representative strains produced a fluorescent compound when visualized under UV light. In contrast colonies cultured from the later three respiratory samples (3, 4 and 5) appeared dry and their representative strains produced little or no fluorescence (Fig. 1B). To determine if all 5 strains isolated over time from this single patient were isogenic, *SpeI* digested genomic DNA from each of the 5 isolates were subjected to PFGE. Under standard PFGE conditions, all 5 clinical isolates appeared clonal (Fig. 1C), suggesting that the decline in patient's clinical status was not due to acquisition of a novel, more virulent strain of *P. aeruginosa*, but to a change in virulence gene expression and/or secretion by the original infecting strain.

We hypothesized that the 5 strains isolated from this patient may represent a set of clonal isolates transitioning from a chronic (strains 1 and 2) to an acute (strains 3, 4 and 5) mode of infection that contributed to the development of VAP in this patient. As previously mentioned, both chronic and acute *P. aeruginosa* infections are typically associated with expression of specific virulence systems, therefore, analysis of these traits was performed for each isolate. Elastase production, typically associated with chronic infections (14) was measured for all 5 isolates. Strains 1 and 2 produced approximately 2-fold higher concentrations of elastase, while strains 3, 4 and 5 did not (Fig. 2A). Measurement of pyoverdine production, the fluorescent siderophore produced by *P. aeruginosa* and co-expressed with other biofilm-associated virulence factors such as alginate and elastase (26), also demonstrated significantly ($p < 0.0001$) increased production by the first two isolates compared to the latter three (Fig. 2B). Examination of biofilm formation by all five strains also demonstrated that the first two strains produced substantially more biofilm than the latter three (Fig. 2C).

To further examine the hypothesis that transition from chronic to acute modes of infection was occurring in these clonal isolates, type III cytotoxin secretion, a hallmark of acute infection (41), was analyzed for all 5 isolates. Both intracellular and secreted fractions were analyzed and demonstrated that while all strains produced the cytotoxins ExoT and ExoU, only the latter 3 isolates secreted them (Fig. 2D). Type III cytotoxins mediate acute lung injury, therefore these observations appeared to correlate with the appearance of bloody ETA samples from this patient as a diagnosis of VAP was made. To confirm that the latter 3 strains were more cytotoxic

compared to the first two strains, *in vitro* cell culture cytotoxicity assays were performed for each strain. Cytotoxicity towards human epithelial cells following a 90 min co-incubation was significantly higher for strains 3, 4 and 5 compared to strains 1 and 2 (Fig. 2E). Collectively, these results supported the hypothesis that during the course of pulmonary infection, a transition from a chronic biofilm mode of virulence to one of planktonic acute, type III mediated pathogenicity occurred in these clonal isolates and moreover that these phenotypes were maintained *in vitro*.

PQS signal concentration regulates secretion of type III cytotoxins

A clue as to how this physiological shift was regulated came from the observation that pyoverdinin production decreased dramatically in the latter 3 strains. Recently, it has been demonstrated that pyoverdinin production is regulated by the quorum sensing molecule PQS (7,13), suggesting that altered concentrations of this signal molecule may be responsible for the physiological changes observed. To examine this hypothesis, an ethyl acetate extract of secreted organic compounds (typically used to purify quinolone signals) was quantified for each strain by TLC, as described in material and methods. As hypothesized, strains 1 and 2 which expressed virulence factors associated with a biofilm mode of lifestyle, produced large concentrations of organic compounds, while the latter 3 type III secreting strains, did not (Fig. 3A). To determine if quinolone signal concentration was specifically affected, Q-PCR analysis of *pqsA* (the first gene in the PQS biosynthetic operon) copy number was performed for all 5 clinical isolates. *pqsA* copy number was on average more than 12-fold higher in the initial two strains compared to the average copy number detected in the latter 3 strains (Fig. 3B), confirming that quinolone signal production decreased substantially in the latter 3 isolates.

To confirm involvement of the PQS biosynthetic cluster in regulation of type III secretion, all isolates were exposed to farnesol, a sesquiterpene produced by many organisms, including *Candida albicans*, and recently identified as an inhibitor of PQS production via promotion of a non-productive binding between PqsR (mfvR) and the *pqsA* promoter (11). Following 4 h of growth in the presence of farnesol (which did not impact culture density), all 5 of the clinical isolates secreted ExoU (Fig. 4A). Notably, the first two isolates, which consistently produced high concentrations of PQS and had not previously demonstrated cytotoxin secretion, exhibited ExoU secretion in the presence of this inhibitor of PQS biosynthesis. To determine specifically which of the two known quinolone signals, HHQ or PQS was responsible for the differential type III secretion observed, purified extracts from PQS-sufficient (clinical strain 1 and PAO1) and deficient (PAO1 Δ *pqsH*) strains were added exogenously to cultures of the 5 clinical isolates. As a control, a set of cultures without exogenous extract was incubated under identical conditions. Western analysis of the secreted fraction of each culture demonstrated that in the absence of exogenously added extract (control), strains exhibited the same profile as previously observed: no cytotoxin secretion by strains 1 and 2, while strains 3, 4 and 5 secreted ExoU (Fig. 4B). Strains exposed to exogenous supernatant extract from PQS-sufficient strains: clinical strain 1 (this study), or PAO1 (to control for molecules that may be secreted specifically by the clinical strain), exhibited no detectible ExoU secretion by any isolate (Fig. 4B), suggesting that high concentrations of a secreted molecule in these supernatants negatively regulated type III cytotoxin secretion. The identity of this signal was confirmed as PQS by the observation that the same strains cultured in the presence of a PQS-deficient extract (purified from PAO1 Δ *pqsH* that produces all other quinolone compounds except PQS), exhibited the same secretion profile as the unexposed control cultures (Fig. 4B). It is pertinent to note that the culture conditions employed for all of these experiments likely results in reduced aeration (see materials and methods), suggesting that PQS regulation of type III cytotoxin secretion may be specific to low oxygen conditions e.g. hypoxic pockets in chronically infected airways in the host.

Discussion

To date, the bacterial signals that regulate transition between reciprocally regulated chronic and acute modes of infection in *P. aeruginosa* are not well understood. While high concentrations of specific quorum sensing signals have previously been shown to induce expression of virulence factors associated with the chronic mode of infection (8), the role of these molecules on the type III secretion system which is active during acute infection is less clear. In this study, a series of ETA samples from a patient developing VAP over a 3-week period provided a collection of clonal *P. aeruginosa* isolates that appeared to transition from a chronic to an acute infection during the interval of observation. Analysis of virulence factors associated with both modes of infection, confirmed that strains of the dominant morphotype isolated initially from this patient exhibited a proclivity for chronic infection, even under culture conditions that induce the TTSS. Conversely, the latter 3 isolates exhibited type III secretion phenotypes consistent with acute infection. Persistence of phenotypic characteristics of chronic or acute virulence gene expression associated with each strain *in vitro* suggests that subtle genomic changes, not identified by standard PFGE e.g. mutations, small-scale chromosomal rearrangements or epigenetic modifications, may have “hard-wired” these strains into the respective mode of infection. This is particularly interesting, given the recent finding that antibiotic administration (which occurred during the course of this patients illness), can induce hypermutability in bacterial strains through reactive oxygen species generation (23), providing a potential explanation for evolution of more virulent sub-populations in otherwise “clonal” strains.

A clue to the nature of the signal that governed this phenotypic switch came from the observation that the first two strains produced substantially more pyoverdine compared to the latter 3 isolates. Pyoverdine is a fluorescent secondary metabolite produced by *P. aeruginosa* which functions as a siderophore and facilitates iron sequestration (10). Recently it has been demonstrated that the quorum sensing signal molecule PQS regulates pyoverdine production (7,13). Examination of crude supernatant extracts from each isolate supported the hypothesis that the concentration of an extracellular product was substantially increased in the initial isolates compared to the latter strains. In addition, inhibition of PQS production (and all other quinolones) by the sesquiterpene farnesol, restored type III secretion phenotype to all strains, suggesting a role for quinolone signaling in controlling TTSS by *P. aeruginosa* under reduced aeration culture conditions. Early isolates that exhibited expression of virulence factors associated with chronic infection produced substantially higher *pqsA* mRNA, compared to the latter isolates that exhibited type III cytotoxin secretion (and produced little or no *pqsA* transcript). Addition of PQS-sufficient or -deficient supernatant extracts to cultures of these clinical isolates, or inhibition of PQS biosynthesis by using farnesol, demonstrated that PQS concentration, either directly or indirectly specifically regulates secretion of type III cytotoxins. Collectively, these observations support the hypothesis that elevated concentrations of PQS promote expression of virulence factors associated with chronic infection while low concentrations or inability to synthesize PQS appear to trigger the reciprocal, acute mode of infection through physiological reprogramming of *P. aeruginosa* that induces motility (1,2) and secretion of type III cytotoxins. This data is supported by a recent study (1) demonstrating that inactivation of the PA2663 gene of *P. aeruginosa* repressed PQS production, leading to a substantial (seven-fold) decrease in elastase activity and induction of genes for motility. Previously we have demonstrated that type III secretion is associated with motile cells that possess a relatively uncomplex lipopolysaccharide (2), which we hypothesized enhanced motility and contact with mammalian cells. These motile cells would, presumably, also be exposed to lower concentrations of PQS, which may explain their proclivity to secrete type III cytotoxins.

Bleves *et al* demonstrated down-regulation of type III secretion using *P. aeruginosa las* and *rhl* mutants, as well as increased secretion of ExoS in a *rhl* mutant strain (6), quorum sensing systems that are known to impact PQS production (12,31). More recently, addition of acylated homoserine lactones (AHL's) has been shown to down-regulate expression, production and supernatant (secreted) LcrV concentrations. LcrV is a key protein in the type III secretion system of *Yersinia pestis* and homolog of the *P. aeruginosa* PcrV protein both of which are located at the tip of the needle complex (18). Conflicting data exists in the literature regarding the role of PQS in regulating *P. aeruginosa* type III cytotoxin gene expression. Shen *et al*, demonstrated that addition of exogenous PQS to cultures of *P. aeruginosa* did not impact expression of the *exsCEBA* operon that encodes the major transcriptional regulators of type III secretion genes (42). However, more recently, Kong *et al*, demonstrated that a *psqR* mutant exhibited significant increases in expression of ExoS and ExoT using a luminescent reporter fused to individual promoters of each of these cytotoxins (24). Shen and colleagues equated expression with secretion, which, based on our data may not be true of *P. aeruginosa* type III secretion. We demonstrate that, in agreement with the Shen study, intracellular concentrations of ExoT and U are relatively unaffected by PQS concentration under our specific culture conditions; all strains demonstrated relatively equivalent intracellular concentrations of these cytotoxins. However, our data quite clearly demonstrates that *secretion* of these cytotoxins is dependent on the concentration of PQS, suggesting that a further level of PQS-mediated post-translational regulation governing cytotoxin translocation exists in *P. aeruginosa*.

The mechanism by which PQS plays into the regulatory circuitry associated with cytotoxin secretion remains to be determined. Given that several two component regulators are known to reciprocally control acute and chronic virulence gene expression by *P. aeruginosa* (47) and are known to respond to environmental signals, it is likely that PQS exerts its effect through at least one of these molecular switches. This hypothesis is bolstered by recent reports that mutation in *retS*, the sensor component of one of these reciprocal switches, attenuates type III virulence (51), coincident with increased exopolysaccharide production (19). Our data generated under conditions of potentially reduced oxygen availability, suggest that PQS may regulate type III secretion under these circumstances. Nonetheless, low oxygen conditions are clinically relevant in lower respiratory tract infections where bronchial obstruction from mucus plugging leads to hypoventilation and diffusion of alveolar oxygen into pulmonary arterioles. In addition, there are several reports of hypoxic pockets in the airways of patients with chronic pulmonary infection, and, moreover that *P. aeruginosa* exists in these mucopurulent niches (49) suggesting that PQS may play a key role in regulating virulence gene expression under these conditions.

The natural history of clinical *P. aeruginosa* isolates in chronic airway infections is reported to involve a loss of type III secretion over time in sequential isolates (22). Our data suggests that strains may also transition in the opposite direction from a "chronic" to an "acute" phenotype under specific conditions. While loss of type III secretion ability appears true of the specific populations studied (22) and may be due to the selection of a dominant biofilm-forming genotype under conditions of repeated antimicrobial administration, it is also possible that very small sub-populations exist within these communities that retain the ability to secrete type III cytotoxins. Certainly there is evidence for phenotypically distinct sub-populations in single-species bacterial biofilms [reviewed in (45)] and the recent demonstration of antimicrobial-induced hypermutability leading to generation of "libraries" of mutants (23) make it highly plausible that small subsets of cells with subtle genotypic distinctions may coexist in these communities. Under the appropriate conditions e.g. when microbial competition is low due to antimicrobial administration, this small subset could potentially become dominant, a strategy that would increase the overall fitness of the population.

That QS molecules play such a key role in regulating virulence factors in *P. aeruginosa*, makes them an attractive target for development of therapeutics. Since the identification of the first furanone compounds produced by the marine algae *Delisia pulchra* that exhibit quorum sensing inhibiting (QSI) activity, many groups have reported natural and synthetic compounds that exhibit this ability (4,20,29,50) and protect against bacterial infection in a murine model (28, 34,50). Human epithelial cells also produce enzymes that interfere with QS (9,38), suggesting that this strategy may be employed as a form of innate immunity against bacterial infection. As a result, quorum sensing inhibitors are being developed as promising novel antimicrobial therapeutics (40). While anti-Pseudomonal QSI compounds may block PQS production and decrease expression of virulence factors associated with a chronic, biofilm lifestyle, there is the possibility that preventing biofilm formation could potentially promote motility and type III cytotoxin secretion. Given our findings, QS-blocking therapies may be most efficacious when used in combination with additional therapeutics e.g. the use of QS inhibitor in combination with a type III blocking therapy and appropriate antimicrobials. Such a combination could force *P. aeruginosa* out of the protective environment of the biofilm and into a free-swimming mode of lifestyle, thus increasing antimicrobial efficacy. Since we have previously demonstrated that these free-swimming cells are also associated with type III secretion, use of an effective anti-type III secretion therapeutic (15,21,35) to prevent acute injury in combination with a QSI and antimicrobial administration may provide an excellent means of resolving recalcitrant chronic infection while preserving epithelial integrity in a number of patient groups. The effects of these types of therapeutic combinations to combat *P. aeruginosa* infections have yet to be determined. What remains clear however, is that the full impact of these emerging species-specific therapeutics must be extensively evaluated in the context of a clinical strain physiology to truly determine their efficacy.

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Bibliography

1. Attila C, Ueda A, Wood TK. PA2663 (PpyR) increases biofilm formation in *Pseudomonas aeruginosa* PAO1 through the psl operon and stimulates virulence and quorum-sensing phenotypes. *Appl Microbiol Biotechnol* 2008;78:293–307. [PubMed: 18157527]
2. Augustin DK, Song Y, Baek MS, Sawa Y, Singh G, Taylor B, Rubio-Mills A, Flanagan JL, Wiener-Kronish JP, Lynch SV. Presence or absence of lipopolysaccharide O antigens affects type III secretion by *Pseudomonas aeruginosa*. *J Bacteriol* 2007;189:2203–9. [PubMed: 17209027]
3. Bassler BL. How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Opin Microbiol* 1999;2:582–7. [PubMed: 10607620]
4. Bjarnsholt T, Jensen PO, Burmolle M, Hentzer M, Haagensen JA, Hougen HP, Calum H, Madsen KG, Moser C, Molin S, Hoiby N, Givskov M. *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology* 2005;151:373–83. [PubMed: 15699188]
5. Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Hoiby N. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol* 2009;44:547–58. [PubMed: 19418571]
6. Bleves S, Soscia C, Nogueira-Orlandi P, Lazdunski A, Filloux A. Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 2005;187:3898–902. [PubMed: 15901720]

7. Bredenbruch F, Geffers R, Nimtz M, Buer J, Haussler S. The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol* 2006;8:1318–29. [PubMed: 16872396]
8. Calfee MW, Coleman JP, Pesci EC. Interference with *Pseudomonas* quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 2001;98:11633–7. [PubMed: 11573001]
9. Chun CK, Ozer EA, Welsh MJ, Zabner J, Greenberg EP. Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *Proc Natl Acad Sci U S A* 2004;101:3587–90. [PubMed: 14970327]
10. Cox CD, Adams P. Siderophore activity of pyoverdinin for *Pseudomonas aeruginosa*. *Infect Immun* 1985;48:130–8. [PubMed: 3156815]
11. Cugini C, Calfee MW, Farrow JM 3rd, Morales DK, Pesci EC, Hogan DA. Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. *Mol Microbiol* 2007;65:896–906. [PubMed: 17640272]
12. Dekimpe V, Deziel E. Revisiting the quorum-sensing hierarchy in *Pseudomonas aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors. *Microbiology* 2009;155:712–23. [PubMed: 19246742]
13. Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong X, Hider RC, Cornelis P, Camara M, Williams P. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* 2007;14:87–96. [PubMed: 17254955]
14. Diggle SP, Winzer K, Chhabra SR, Worrall KE, Camara M, Williams P. The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* 2003;50:29–43. [PubMed: 14507361]
15. Faure K, Fujimoto J, Shimabukuro DW, Ajayi T, Shime N, Moriyama K, Spack EG, Wiener-Kronish JP, Sawa T. Effects of monoclonal anti-PcrV antibody on *Pseudomonas aeruginosa*-induced acute lung injury in a rat model. *J Immune Based Ther Vaccines* 2003;1:2. [PubMed: 12943554]
16. Fletcher MP, Diggle SP, Camara M, Williams P. Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules. *Nat Protoc* 2007;2:1254–62. [PubMed: 17546021]
17. Fothergill JL, Panagea S, Hart CA, Walshaw MJ, Pitt TL, Winstanley C. Widespread pyocyanin overproduction among isolates of a cystic fibrosis epidemic strain. *BMC Microbiol* 2007;7:45. [PubMed: 17521417]
18. Gelhaus HC, Rozak DA, Nierman WC, Chen D, Varga JJ, Zadeh M, Ulrich RL, Adamovicz JJ. Exogenous *Yersinia pestis* quorum sensing molecules N-octanoyl-homoserine lactone and N-(3-oxooctanoyl)-homoserine lactone regulate the LcrV virulence factor. *Microb Pathog* 2009;46:283–7. [PubMed: 19249344]
19. Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* 2004;7:745–54. [PubMed: 15525535]
20. Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Hoiby N, Givskov M. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *Embo J* 2003;22:3803–15. [PubMed: 12881415]
21. Imamura Y, Yanagihara K, Fukuda Y, Kaneko Y, Seki M, Izumikawa K, Miyazaki Y, Hirakata Y, Sawa T, Wiener-Kronish JP, Kohno S. Effect of anti-PcrV antibody in a murine chronic airway *Pseudomonas aeruginosa* infection model. *Eur Respir J* 2007;29:965–8. [PubMed: 17301098]
22. Jain M, Bar-Meir M, McColley S, Cullina J, Potter E, Powers C, Prickett M, Seshadri R, Jovanovic B, Petrocheilou A, King JD, Hauser AR. Evolution of *Pseudomonas aeruginosa* type III secretion in cystic fibrosis: a paradigm of chronic infection. *Transl Res* 2008;152:257–64. [PubMed: 19059160]
23. Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell* 2010;37:311–20. [PubMed: 20159551]

24. Kong W, Liang H, Shen L, Duan K. [Regulation of type III secretion system by Rhl and PQS quorum sensing systems in *Pseudomonas aeruginosa*]. *Wei Sheng Wu Xue Bao* 2009;49:1158–64. [PubMed: 20030052]
25. Kuchma SL, Connolly JP, O'Toole GA. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* 2005;187:1441–54. [PubMed: 15687209]
26. Laux DC, Corson JM, Givskov M, Hentzer M, Moller A, Wosencroft KA, Olson JC, Krogfelt KA, Goldberg JB, Cohen PS. Lysophosphatidic acid inhibition of the accumulation of *Pseudomonas aeruginosa* PAO1 alginate, pyoverdine, elastase and LasA. *Microbiology* 2002;148:1709–23. [PubMed: 12055291]
27. Le Berre R, Nguyen S, Nowak E, Kipnis E, Pierre M, Ader F, Courcol R, Guery BP, Faure K. Quorum-sensing activity and related virulence factor expression in clinically pathogenic isolates of *Pseudomonas aeruginosa*. *Clin Microbiol Infect* 2008;14:337–43. [PubMed: 18190582]
28. Lesic B, Lepine F, Deziel E, Zhang J, Zhang Q, Padfield K, Castonguay MH, Milot S, Stachel S, Tzika AA, Tompkins RG, Rahme LG. Inhibitors of pathogen intercellular signals as selective anti-infective compounds. *PLoS Pathog* 2007;3:1229–39. [PubMed: 17941706]
29. Manefield M, de Nys R, Kumar N, Read R, Givskov M, Steinberg P, Kjelleberg S. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* 1999;145(Pt 2):283–91. [PubMed: 10075410]
30. Manos J, Arthur J, Rose B, Tingpej P, Fung C, Curtis M, Webb JS, Hu H, Kjelleberg S, Gorrell MD, Bye P, Harbour C. Transcriptome analyses and biofilm-forming characteristics of a clonal *Pseudomonas aeruginosa* from the cystic fibrosis lung. *J Med Microbiol* 2008;57:1454–65. [PubMed: 19018014]
31. McGrath S, Wade DS, Pesci EC. Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). *FEMS Microbiol Lett* 2004;230:27–34. [PubMed: 14734162]
32. Mikkelsen H, Bond NJ, Skindersoe ME, Givskov M, Lilley KS, Welch M. Biofilms and type III secretion are not mutually exclusive in *Pseudomonas aeruginosa*. *Microbiology* 2009;155:687–98. [PubMed: 19246740]
33. Mittal R, Sharma S, Chhibber S, Harjai K. Contribution of quorum-sensing systems to virulence of *Pseudomonas aeruginosa* in an experimental pyelonephritis model. *J Microbiol Immunol Infect* 2006;39:302–9. [PubMed: 16926976]
34. Miyairi S, Tateda K, Fuse ET, Ueda C, Saito H, Takabatake T, Ishii Y, Horikawa M, Ishiguro M, Standiford TJ, Yamaguchi K. Immunization with 3-oxododecanoyl-L-homoserine lactone-protein conjugate protects mice from lethal *Pseudomonas aeruginosa* lung infection. *J Med Microbiol* 2006;55:1381–7. [PubMed: 17005787]
35. Neely AN I, Holder A, Wiener-Kronish JP, Sawa T. Passive anti-PcrV treatment protects burned mice against *Pseudomonas aeruginosa* challenge. *Burns* 2005;31:153–8. [PubMed: 15683685]
36. Nicas TI, Iglewski BH. Production of elastase and other exoproducts by environmental isolates of *Pseudomonas aeruginosa*. *J Clin Microbiol* 1986;23:967–9. [PubMed: 3086372]
37. Nicas TI, Iglewski BH. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. *Can J Microbiol* 1985;31:387–92. [PubMed: 2988728]
38. Ozer EA, Pezzulo A, Shih DM, Chun C, Furlong C, Lulis AJ, Greenberg EP, Zabner J. Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing. *FEMS Microbiol Lett* 2005;253:29–37. [PubMed: 16260097]
39. Pearson JP, Feldman M, Iglewski BH, Prince A. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. *Infect Immun* 2000;68:4331–4. [PubMed: 10858254]
40. Rasmussen TB, Givskov M. Quorum-sensing inhibitors as anti-pathogenic drugs. *Int J Med Microbiol* 2006;296:149–61. [PubMed: 16503194]
41. Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW, Wiener-Kronish JP. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* 2001;183:1767–74. [PubMed: 11372029]

42. Shen DK, Filopon D, Chaker H, Boullanger S, Derouazi M, Polack B, Toussaint B. High-cell-density regulation of the *Pseudomonas aeruginosa* type III secretion system: implications for tryptophan catabolites. *Microbiology* 2008;154:2195–208. [PubMed: 18667553]
43. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 2000;407:762–4. [PubMed: 11048725]
44. Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A, Palacios S, Manoil C, Kirisits MJ, Starner TD, Wozniak DJ, Harwood CS, Parsek MR. *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J Bacteriol* 2009;191:3492–503. [PubMed: 19329647]
45. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. *Nat Rev Microbiol* 2008;6:199–210. [PubMed: 18264116]
46. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 2000;406:959–64. [PubMed: 10984043]
47. Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S, Lazdunski A, Lory S, Filloux A. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* 2006;103:171–6. [PubMed: 16373506]
48. Wade DS, Calfee MW, Rocha ER, Ling EA, Engstrom E, Coleman JP, Pesci EC. Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* 2005;187:4372–80. [PubMed: 15968046]
49. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Doring G. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 2002;109:317–25. [PubMed: 11827991]
50. Wu H, Song Z, Hentzer M, Andersen JB, Molin S, Givskov M, Hoiby N. Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. *J Antimicrob Chemother* 2004;53:1054–61. [PubMed: 15117922]
51. Zolfaghar I, Angus AA, Kang PJ, To A, Evans DJ, Fleiszig SM. Mutation of *retS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa*, attenuates multiple virulence mechanisms. *Microbes Infect* 2005;7:1305–16. [PubMed: 16027020]

Fig. 1.
Fig. 1A. Serial ETA's collected over a 3 week period from a patient developing VAP exhibit evidence of a transition from chronic (green tinged appearance) to acute (bloody appearance) mode of infection. **B.** *P. aeruginosa* isolates demonstrate a shift in fluorescent metabolite production upon exposure to UV light. **C.** Serial isolates are clonal by standard PFGE analysis.

Fig. 2.

Fig. 2A. Elastase production is approximately 2-fold higher in strains 1 and 2 compared to strains 3, 4 and 5. **B.** Pyoverdine production is also greater in the initial two isolates compared to the latter 3. **C.** Biofilm formation by all five strains. **D.** Analysis of intracellular (IC) and secreted total protein fractions (S) of each strain demonstrates that while all strains produce ExoU and ExoT, only the latter 3 isolates secrete these cytotoxins. **E.** Cytotoxicity assays profiling epithelial cell toxicity of all 5 strains demonstrate that the latter 3 isolates are significantly more cytotoxic compared to strains isolated earlier in the infectious process.

Fig. 3.

Fig. 3A. Substantially higher concentrations of extracellular organic compounds (including quinolone signals) in cell-free extract are produced by strains 1 and 2, compared to strains 3, 4 and 5. **B.** Q-PCR analysis confirms that strains 1 and 2 express approximately 12-fold higher *pqsA* copy number compared to strains 3, 4 and 5.

Fig. 4.

Fig. 4A. Inhibition of AHQ biosynthesis by farnesol addition induces a type III secretion phenotype in all five isolates. **B.** Strains cultured without exogenous extract exhibit cytotoxin secretion by the latter 3 strains (Control), those exposed to PQS-containing extract (PQS⁺) obtained from clinical strain 1 (this study) or the laboratory strain PAO1 exhibit no evidence of cytotoxin secretion, while strains cultured in the presence of extract from PAO1Δ*pqsH* exhibit a secretion profile identical to the control cultures, confirming the role of PQS in type III cytotoxin secretion.

Table 1

Clinical isolates used in this study and patient status at time of sample collection.

Strain number	Date of isolation	Colony Appearance	VAP development	
			Sputum quantity and appearance	X-ray findings
1	12/10/'04	Glossy	Little; non-purulent	Patchy consolidation
2	12/14/'04	Glossy	Unknown	Increased consolidation
3	12/25/'04	Dry	Large; purulent	Diffuse infiltrate
4	12/28/'04	Dry	Moderate; purulent	Diffuse infiltrate
5	01/02/'05	Dry	Moderate; purulent	Diffuse infiltrate