Review

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The Pseudomonas Quinolone Signal (PQS)

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Abstract: *Pseudomonas aeruginosa* is an opportunistic human pathogen that routinely appears near the top of public health threat lists worldwide. *P. aeruginosa* causes infections by secreting a wealth of exceptionally active exoproducts, leading to tissue damage. The synthesis of many of these virulence factors is now known to be under the control of the quorum sensing (QS) system. Over the last 15 years, the *Pseudomonas* quinolone signal (PQS) has been found to play a crucial role in QS by linking the two segments (las and rhl) of the *P. aeruginosa* *N*-acylhomoserine lactone-dependent QS signaling pathways. Herein, we present the discovery and elucidation of PQS signaling from a historical perspective, and also outline some of the outstanding research questions that still need to be addressed. Finally, we show how a better understanding of the biochemistry underpinning this pathway is leading to the development of new antimicrobial interventions with clear therapeutic potential.

Keywords: antimicrobial agents · biological activity · *Pseudomonas* quinolone signal · quorum sensing · virulence

1 Introduction

Since its discovery and initial characterization some 15 years ago by Pesci *et al.*,[1] one particular class of *Pseudomonas aeruginosa* intercellular signaling molecules, the 4-alkylquinolones (AQs), and the genes involved in the synthesis of these molecules, have appeared with refreshing regularity in many studies involving this organism. Herein, we present a historical overview of how alkyl quinolones and their signaling pathways were discovered, what these molecules do, what gaps remain in our knowledge, and how researchers are manipulating AQ signaling for potential therapeutic benefit. The review is not intended to be exhaustive or detailed – the reader is referred to several other excellent monographs for those purposes;[2–4] instead, it is intended to provide a "taster" of the field and some insights into unresolved questions.

2 The Clinical Significance of *P. aeruginosa*

To date, AQ signaling has been almost exclusively associated with *P. aeruginosa*, although a similar type of signaling molecule has also been identified in certain *Burkholderia sp.*[5] *P. aeruginosa* is a major cause of nosocomial infections and is also associated with a large number of chronic infections and basal pathologies, especially among immune-compromised individuals and patients with cystic fibrosis (CF)-associated airway infections. It is still not clear why the latter are so exquisitely susceptible to *P. aeruginosa* infection. However, by their late 'teens, the airways of most CF patients show signs of chronic colonization by *P. aeruginosa* or (more rarely) *Burkholderia sp.*

P. aeruginosa is renowned for secreting large quantities of exceptionally active tissue-degrading exoenzymes (proteases, phospholipases, etc.) During acute infections, it is the tissue-macerating activity of these enzymes that causes damage to the patient. AQ signaling plays an important role in stimulating the production of exoenzymes and other tissue-damaging exoproducts. Consequently, if AQ signaling is blocked, virulence should be attenuated, making AQ signaling a key target for the development of anti-pseudomonal therapeutic interventions. However, the same logic does not necessarily apply to chronic infections, such as those associated with the CF airways. During chronic infection, exoenzyme production is dampened down due to the accumulation of mutations in the master regulator(s) of virulence,[6,7] and ongoing tissue damage is mostly thought to be caused by the exuberant host inflammatory response (itself elicited by the continu-

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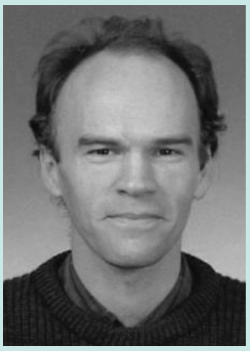
al presence of high titers of bacteria in the lung tissue). This notwithstanding, AQs may still play an important role in such infections because they promote biofilm formation. Biofilms are aggregates of cells encased in a polysaccharide matrix that exhibit enhanced resistance to antibiotics and the host immune response. There is evidence to suggest that *P. aeruginosa* may predominantly exist as biofilms in the airway secretions of many CF patients.[8,9]



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Moreover, AQs can be found in abundance in some of these samples.[10] Consequently, AQ-targeted interventions may give the immune system the upper hand in staving off chronic *P. aeruginosa* infection, possibly for several years. If successful, in addition to their economic benefit, such approaches would lead to improved quality of life and increased lifespan, since the decline in CF lung function is directly linked to *P. aeruginosa* infection.[11]

3 Quorum Sensing in *P. aeruginosa*: Development of an Early Model

Quorum sensing (QS) is a form of intercellular bacterial communication involving diffusible signaling molecules.[12] QS was first characterized in the mid-1970s as mechanism controlling bioluminescence in the marine organism *Vibrio fischerii* (reviewed in refs. [13] and [14]). Originally considered to be an interesting but rather quirky gene regulatory mechanism, QS remained consigned to the backwaters of mainstream microbiology research until the early–mid-1990s, when it became clear that a number of important pathogens (including *P. aeruginosa*) used the same general mechanism to control a range of pathogenicity-associated phenotypes. By the end of that decade, a widely accepted simplistic model had developed, which posited that bacteria continually secreted QS-signaling molecules (which, at that time, were primarily thought to be *N*-acylated homoserine lactones, AHLs). AHLs are freely cell-permeable, such that their intracellular concentration directly reflects the bulk concentration of the molecules in the culture as a whole. Consequently, as the population cell density increases due to cell division and growth, so too does the AHL concentration. The catch is that the cells also express a LuxR-type intracellular receptor for AHLs (with different organisms expressing receptors with specificity for their "own" cognate AHL molecules). Once the intracellular concentration of the AHL exceeds a certain threshold value (presumably related to the K_d of its cognate LuxR homologue for that AHL and the affinity of the AHL–LuxR complex for its target promoter(s)), the LuxR homologue becomes activated, either through conformational change per se, or through conformational change leading to dimerization of the LuxR homologue. LuxR-type proteins are transcriptional regulators and, upon ligand-dependent activation, they bind to "lux boxes" upstream of target genes, leading to an increase (or rarely, also a decrease) in the expression of those genes.

The QS system in *P. aeruginosa* is slightly more complex than most in that it consists of two hierarchical but interlinked AHL-producing systems (reviewed in ref.[15]). At the top of the hierarchy is the las signaling system. Here, LasI is an AHL synthase that generates 3-oxododecanoyl-L-homoserine lactone (OdDHL). This is recognized by its cognate LuxR homologue, LasR. In the activated state, LasR stimulates 1) further expression of *lasI* (thereby amplifying the rate of signal generation) and 2) the expression of a subset of virulence-related genes, including the major elastase LasB (from whence the las pathway derived its moniker). In addition, the LasR-OdDHL complex also stimulates the expression of *rhlR* (encoding another LuxR homologue), which, in turn, also stimulates expression of the adjacent rhlI gene (encoding another AHL synthase which makes butanoyl-L-homoserine lactone (BHL)). In this way, BHL rapidly accumulates in the culture and is continually titrated by RhlR. In the activated form, RhlR not only positively autoregulates BHL production (by stimulating *rhlI* expression), it also promotes the expression of another subset of virulence genes, including the rhamnolipid genes (from which the rhl subsystem gained its name). Layered on top of (or feeding into) this "core" QS machinery are various other regulators,[16,17] many of which impinge upon expression of one or more of the signaling components, thereby adding subtlety and flexibility to the pathway(s). Reinforcing the importance of the las and rhl signaling pathways, mutants defective in either were found to be avirulent,[18] presumably because they expressed far fewer virulence determinants than the wild-type. Remarkably, the same mutants also displayed much reduced biofilm formation. These observations led to the idea that by blocking QS (e.g., by targeting AHL binding to LasR or RhlR) one could deliver a "double whammy" by simultaneously 1) abrogating virulence, and 2) reducing biofilm formation, thereby making *P. aeruginosa* infections less aggressive and easier to clear, especially in combination with "conventional" antibiotic intervention. Indeed, early work in this regard was promising,[19,20] and, although recent findings have somewhat dampened early optimism about the generic utility of QS blockers,[21] it is still clear that QS inhibition may still have therapeutic potential.[22] This was the general state-of-play in the field by the close of the last millennium. However, and as is often the case in science, in spite of the elegance of the *P. aeruginosa* QS model outlined above (and the enthusiastic support it generally received from the research community), new findings revealed that the mechanism(s) underpinning intercellular communication was still far from being "understood".

4 A Historical Perspective: The Discovery of AQ Signaling

In 1999, Pesci *et al.*[1] made an important observation. They found that the addition of spent culture supernatant from wild-type cells of the type strain PAO1 was able to greatly stimulate the expression of a *lasB'-lacZ* reporter construct in a *lasR* mutant. This was unexpected, since, in the absence of *lasR*, both the las and rhl signaling pathways should be inactive or minimally active, respectively.

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Furthermore, addition of purified OdDHL or BHL (or both together) did not elicit the same degree of stimulation as the wild-type culture supernatant did. These findings strongly hinted that a third, as-yet uncharacterized, but highly potent, QS signaling molecule might be present. The same wild-type culture supernatant did not stimulate *lasB* expression in a *lasI rhlI* double mutant, suggesting that LasR might be required for the bioactivity of the new signal. However, additional experiments indicated that LasR was unlikely to be the receptor for the new signaling molecule, indicating that, regardless of the nature of the signal, it probably interfaces with the AHL-dependent QS system at some point between the las and rhl pathways. Subsequent fractionation of the wild-type spent culture supernatant (and detailed chemical analyses of the bioactive fractions) revealed that the new signaling molecule was an alkyl quinolone, 2-heptyl-3-hydroxy-4-quinolone, or *Pseudomonas* quinolone signal (PQS; Figure 1).

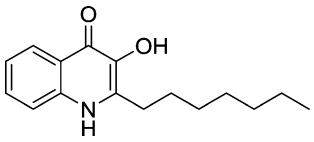

Figure 1. Chemical structure of PQS.[1]

In parallel with Pesci *et al.*, Rahme and colleagues had been attempting to identify virulence-defective mutants in a different, more virulent strain of *P. aeruginosa*, PA14.[23] These researchers identified a genetic locus that, when disrupted through transposon (Tn) insertion, yielded a mutant that was avirulent in plants and animals, and produced only low levels of secreted exoenzymes and the secondary metabolite pyocyanin. Mapping of the Tn insertion revealed that it had disrupted an open reading frame (ORF) that was subsequently denoted *mvfR* (multiple virulence factor regulator; although this ORF is more widely known by the name ascribed to it in PAO1, *pqsR*). The expression of *mvfR* was independent of the two AHL-dependent QS regulators, LasR and RhlR. This indicated that, although MvfR regulated multiple QS-dependent components, it apparently functioned independently of the known regulators of the QS circuit. Sequence analysis revealed that MvfR was a member of the ligand-activated LysR-type transcriptional regulators, with a helix-turn-helix DNA-binding domain at the N terminus and a ligand-binding domain at the C terminus. The question was which genes were regulated by MvfR? Rahme *et al.* provided some early insight here by noting that MvfR was located adjacent to the *phnAB* operon. The *phnAB* genes encode an anthranilate synthase; chorismate-derived anthranilate was originally thought to be a precursor of pyocyanin. Given that the *mvfR* mutant was deficient in pyocyanin production, they postulated that MvfR might have regulated the *phnAB* operon. This indeed turned out to be the case. It is interesting to note at this point that *P. aeruginosa* encodes two anthranilate synthases: PhnAB and TrpEG. Of these, only *phnAB* is regulated by MvfR.

While Rahme and colleagues were working on the regulation of MvfR and the discovery of MvfR ligands in PA14, Manoil's lab were identifying genes involved in the production of PQS.[24] They did this by utilizing the fact that production of the bright-blue pigment produced by *P. aeruginosa*, pyocyanin is tightly controlled by PQS. A set of Tn mutants defective in pyocyanin production were isolated and characterized in detail. This clutch of mutants reassuringly contained Tn insertions in the pyocyanin biosynthetic genes (*phzM*, *phzS*, and *phzA1-E1*) as well as a number of known pyocyanin regulators, such as *lasR* and *rhlIR*, and also a set of uncharacterized ORFs. Given the known link between pyocyanin and PQS, the pyocyanin-deficient mutants were also screened for PQS production. Most displayed wild-type or marginally reduced PQS levels. However, some produced no PQS at all. Interestingly, the Tn insertions in these PQS deficient mutants were almost all located in a single region: PA0996–PA1003. Within this region, genes PA0996–PA1000 formed a polycistronic operon (now known as the *pqsA-E* operon) and PA1001–PA1002 formed a second, convergent operon (the *phnAB* operon). As noted earlier, the *phnAB* operon is followed immediately by *mvfR* (PA1003, denoted *pqsR* in PAO1). Unlike most of the pyocyanin-deficient mutants examined (which retained full lethality in an animal infection model), the mutants containing insertions in this gene cluster displayed greatly reduced virulence *in vivo*. The fact that *phnAB* mutants were defective in PQS biosynthesis indicated that anthranilate was, in fact, a precursor for PQS biosynthesis, rather than a precursor of pyocyanin biosynthesis. Independent work by Calfee *et al.*[25] confirmed this conclusion, showing that radiolabelled anthranilate yielded radiolabelled PQS. That study also highlighted the potential of PQS signaling as a target for antimicrobial development: methyl anthranilate inhibited PQS production and also diminished *lasB* expression in vivo. Given that Rahme's lab had already shown that MvfR regulated *phnAB* expression,[23] these observations suggested that MvfR (PqsR) might control PQS biosynthesis. One additional mutation that gave rise to PQS deficiency was located outside of the *pqs* operon in a gene now denoted *pqsH*. Gallagher *et al.* showed that *pqsH* expression was regulated by LasR,[24] thereby linking PQS synthesis with the AHL-dependent las signaling pathway.

Not all mutations in the *pqs* gene cluster abolished PQS production. Mutants carrying insertions in *pqsE* produced wild-type levels of PQS, but remained avirulent. This suggested that PqsE was not involved in the synthesis of PQS, but did play a role in the response to this compound: it is the principle *effector* of PQS signaling.

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Weight was leant to the significance of PQS in pathogenicity by the discovery (in 2002) by Collier *et al.*[10] that PQS was abundant in the airway secretions of patients with CF who were infected with *P. aeruginosa*. In parallel, work from the Williams and Iglewski labs[26,27] showed that PQS was maximally produced during the stationary-phase growth, and that PQS signaling controlled the rhl branch of the AHL-dependent QS system. Indeed, mutants in *pqsR* (defective in PQS synthesis) or *pqsE* (defective in the response to PQS) displayed reduced production of virulence factors, but retained wild-type levels of BHL production.[26] The same team also showed that PQS could be produced (albeit with delayed kinetics) in the absence of LasR. This work led to the emergence of a model that placed PQS at the interface between the las and rhl signaling pathways.

By 2004, PQS had firmly established itself as an important signaling molecule, and the genes involved in its biosynthesis and response had been tentatively identified. However, the signal still lacked a receptor and the biosynthetic pathway had yet to be elucidated. The latter problem was investigated by Déziel *et al.*,[28] who proposed that PQS synthesis was directed in two distinct steps (a suggestion subsequently confirmed and extended by others[29,30]). The PqsA-D proteins catalyze the condensation of β-keto fatty acids with anthranilate to yield 2-alkyl-4-quinolones (AHQs), prominent among which is 2-heptyl-4(1*H*)-quinolone (HHQ; Figure 2). Déziel *et al.*

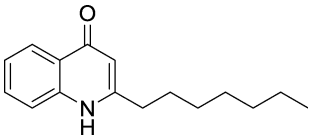

Figure 2. Chemical structure of HHQ.

also provided evidence that HHQ was a diffusible molecule that could be produced in one cell and then converted into PQS in another through the action of PqsH, a mono-oxygenase.[28] Because of this, it has been proposed that HHQ serves as a "messenger" molecule rather than a signal *per se*. The same team also inferred that MvfR (PqsR) positively regulated transcription of the *pqsA-E* operon. By 2005, Rahme's team were investigating this possibility in detail.[31] Microarray-based comparison of the global transcriptional profile of wild-type cells versus *mvfR* mutant cells revealed that not only was expression of the *pqs*/*phn* operons abolished in the *mvfR* mutant, so too was the expression of the phenazine biosynthetic genes and several other virulence factors (although, notably, some key virulence-associated genes that one would expect to be modulated, such as *lasB*, were unaffected in the microarray analysis). In addition, expression of the *mexGHI-opmD* multi-drug efflux pump was abolished in the *mvfR* mutant. These researchers also showed that mutants unable to make PQS (*pqsA* mutants) or respond to PQS (*pqsE* mutants) displayed greatly reduced virulence in a mouse burn/infection model. These data indicated that the lower virulence associated with *mvfR* mutants was probably due to loss of *pqsE* expression, rather than loss of PQS production *per se*.

At the same time as Rahme's team were quantifying the global impact of PQS signaling, Pesci's lab[32] was using direct biochemical assays to show that PqsR directly bound to the promoter of the *pqs* operon. This binding was increased in the presence of PQS itself, which strongly suggested that PQS might be the co-inducer and that PqsR was a receptor for this molecule. Furthermore, they found that *pqsR* expression was stimulated by LasR and repressed by RhlR, creating a feedback system in which PqsR levels were held in check by the two arms of the AHL-dependent QS system. Xiao *et al.*[33] extended these findings by showing that PqsR (MvfR) bound to a "LysR box" centered 45 base pair (bp) upstream of the *pqsA-E* transcriptional start site (TSS). They also found that LasR bound to a *las*/*rhl* box 513 bp upstream of the TSS and that RhlR bound to a different *las*/*rhl* box located 311 bp upstream of the TSS. Adding further nuance to an already complex regulatory system, very recent evidence (published in December 2014) suggested that RhlR was able to stimulate expression of an alternative *pqsA-E* transcript, in which the Shine-Dalgarno sequence (ribosome binding site) was occluded within a hairpin loop. It seems then that the rhl pathway acts to damp down PQS signaling on several distinct levels.[34]

At this point, some six to seven years after it was first discovered, PQS still lacked a receptor, although it seemed increasingly likely that PqsR (MvfR) might fulfill this role. This issue was again addressed by Rahme's lab.[35] They found that MvfR (PqsR) had dual ligands; the protein binds both HHQ and PQS, and both ligands stimulate MvfR binding to the *pqsA-E* promoter region. They also found that both HHQ and PQS were able to bind to the purified ligand-binding domain of MvfR, eliciting a change in conformation of the protein. This change in conformation manifested itself as a decrease in protein solubility, allowing a rudimentary quantitative comparison of ligand affinities to be derived; PQS bound MvfR more avidly than HHQ. This binding differential was also reflected in the potency of these ligands in stimulating *pqsA-E* transcription; PQS was about 100-fold more active than HHQ. Interestingly, and in the same study, Xiao *et al.* also showed that, in a burned mouse model of infection, a PA14 *pqsH* mutant was as virulent as the wild-type (in contrast, *mvfR* or *pqsA* mutants were avirulent). These observations strongly suggested that HHQ, not PQS, was the main mediator of pathogenicity in *P. aeruginosa in vivo*. The apparently incomplete conversion of HHQ into PQS in both the burned mouse model and in infected human tissue is curious.[36] Given the much higher intrinsic bioactivity of PQS compared

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with HHQ, this observation currently lacks a clear explanation, especially given that other workers have found that it is PQS, not HHQ, that is required for optimal activation of certain target genes.[37] A trivial explanation is that the PA14 strain used by Rahme *et al.* behaves differently *in vivo* compared with the more "domesticated" PAO1 used by most other laboratories. Interestingly, there is also evidence to suggest that the binding of PQS or HHQ to PqsR causes the protein to become differentially proteolytically processed *in vivo*.[23] This processing appears to alter the subcellular localization of the protein, although, to the best of our knowledge, there has been no follow-up work to investigate the consequences (if any) of this.

By 2006, a basic model for PQS signaling had been developed, which has not progressed much since that time. The las signaling system stimulates expression of *pqsR* and *pqsH*. The resulting PqsR protein binds upstream of the *phnAB* and *pqsA-E* operons, thereby stimulating the synthesis of HHQ, which, in turn, is converted to PQS by PqsH. The upregulation of *pqsA-E* yields more HHQ/PQS, thereby setting up a positive feedback loop. In addition to upregulating HHQ production, the increased expression of the *pqsA-E* operon gives rise to increased levels of PqsE, the main effector of the PQS pathway. More recent work by Pesci's team[38] showed that PqsE impinged somehow on the rhl signaling pathway, possibly by synergizing RhlR activity. PqsE is a member of the metallo-β-lactamase family of proteins, and may therefore be responsible for generating a low-molecular-weight metabolite, which could alter RhlR function, although whether the protein actually possesses enzyme activity is still an open question. However, the tantalizing possibility that PqsE does act in this way opens up additional avenues by which PQS signaling might be blocked. Finally, and to prevent a runaway positive feedback loop from draining the cell's metabolic resources, the RhlR protein also acts to damp down the PQS signaling pathway by repressing *pqsA-E* expression.

5 New Twists and Turns to the Story

PQS is more hydrophobic than HHQ, which may go some way towards explaining why HHQ should be the diffusible "messenger" molecule (being converted by PqsH into the more bioactive species, PQS, once safely inside the recipient cell[28,37]). However, *P. aeruginosa* seems to have evolved a more direct solution to the problem of PQS trafficking. It turns out that the supernatant of *P. aeruginosa* cultures contains large amounts of PQS, but not in a "free" soluble form. Instead, the molecule is packaged up in secreted membrane vesicles.[39] Depletion of these vesicles from the supernatants abolished their PQS-associated bioactivity. Remarkably, the same team also found that PQS was responsible for stimulating vesicle formation, indicating that PQS mediated its own packaging. HHQ did not do this. PQS is able to stimulate membrane vesicle formation by interacting with the acyl chains and 4′-phosphate of bacterial LPS, leading to outer membrane blebbing.[40] The membrane vesicles thus generated are able to fuse with the outer membrane of nearby recipient bacteria, where they deposit their cargo (which includes not only PQS, but also a number of proteins and small molecules, including other AQs, too). In a similar vein, Pesci's lab has also looked at other ways in which PQS solubility may be increased *in vivo*. They found that rhamnolipids (biodetergents, the synthesis of which is largely determined by the rhl arm of the AHL-dependent QS system) increase PQS solubility and bioactivity.[41]

As noted earlier, in the microarray analysis of Déziel *et al.*,[31] one of the gene clusters that is downregulated in an *mvfR* mutant encodes the MexGHI-OpmD multi-drug efflux pump. However, subsequent work from Newman's lab suggested that *mexGHI-opmD* was actually regulated by pyocyanin, and that the lack of this phenazine pigment in the *mvfR* mutant most likely accounted for the observed effects on *mexGHI-opmD* transcription.[42] Nevertheless, MexGHI-OpmD has been indirectly implicated in the regulation of PQS-dependent phenotypes. Aendekerk *et al.* found that mutation of the *mexGHI-opmD* genes resulted in an inability to produce PQS (as well as OdDHL), leading to impaired virulence and growth.[43] Provision of exogenous PQS largely restored the wild-type phenotype in these mutants. Further analysis revealed that, in the *mexGHI-opmD* mutants, anthranilate (the precursor of PQS, made by PhnAB) accumulated to toxic levels. Consistent with this, the growth-inhibited phenotype of a *mexI* mutant was relieved when *phnA* (encoding the enzyme responsible for anthranilate synthesis) was also mutated.

PQS is a quixotic molecule and can potentially mediate biological effects through several routes. For example, around 140 genes are known to be controlled by MvfR,[31] including several known virulence factors. However, not all of these effects are necessarily directly controlled by PQS (or HHQ) binding to MvfR. As noted above, *mexGHI-opmD* expression is regulated as a consequence of downstream pyocyanin signaling (which is abrogated in the *mvfR* mutant). Moreover, Bredenbruch *et al.* found that when wild-type cells were treated with PQS, many genes known to be stimulated by iron deprivation (e.g., the pyochelin cluster and certain pyoverdine biosynthetic genes) were expressed.[44] One explanation for this is that AQs can bind iron exceptionally tightly; the pFe^{3+} of the water-soluble 2-methyl-3-hydroxy-4-quinolone (MPQS) iron(III) complex is 16.6. The iron-chelating properties of PQS are probably due to the 3-hydroxy-pyridine-4-one moiety (indeed, such quinolone molecules have been routinely used for many years in research laboratories to "deferrate" media and buffers, etc.) It has been suggested

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that PQS treatment mimics iron starvation because the AQ traps iron in a non-deliverable form.[37] Indeed, when large volumes of stationary-phase LB-grown *P. aeruginosa* cultures are sedimented, the cell pellet is often accompanied by a small amount of very dense dark material; this is the insoluble PQS–iron complex.[45] The cell senses this iron depletion and, in response, upregulates the expression of siderophores to compensate by more effectively scavenging available iron. Although an elegant explanation for the data of Bredenbruch *et al.*, there is probably (much) more to it than this. For example, Rampioni *et al.* recently showed that the expression of siderophore-associated genes in *P. aeruginosa* was also strongly PqsE-dependent, indicating that iron trapping by PQS alone did not account for the full extent of siderophore gene transcriptional stimulation.[46] Hazan *et al.* also noted that PQS lost biological activity (i.e., the ability to stimulate *pqsA-E* transcription) when complexed with iron, and suggested that virulence was likely to be fine-tuned by the balance between free iron and PQS levels.[47] Interestingly, iron-chelated PQS is capable of catalyzing the formation of hydroxyl free radicals ($^{\bullet}$OH). This may explain why Bredenbruch *et al.* also noted that provision of exogenous PQS to cultures upregulated the expression of many genes involved in the oxidative stress response.[44] In subsequent work, Häussler and Becker went further by suggesting that PQS might be involved in shaping the overall *P. aeruginosa* population structure through a combination of pro-oxidative effects (mediated by the free radical inducing Fe^{3+}–PQS complex) and antioxidative effects (unliganded PQS is a powerful reducing agent).[48]

The microarray analysis of Bredenbruch *et al.* (comparing PAO1 grown with or without exogenously added PQS[44]) and Déziel *et al.* (comparing wild-type PA14 with an isogenic *mvfR* mutant[31]) also provided further mechanistic insights into PQS signaling. Both of these studies revealed that transcription of *rsmA*, encoding a potent post-transcriptional regulator known to be involved in the control of virulence,[49] was upregulated by PQS. These observations suggest that PQS can indirectly affect gene expression through post-transcriptional effects. On a final note, Bredenbruch *et al.* also found that *rhlR* expression increased in response to low iron concentrations, indicating that at least some of the effects of PQS on downstream rhl signaling might be mediated through its effect(s) on iron depletion.[44]

Alkyl quinolones may help to protect the *P. aeruginosa* niche from competitors too. As shown by Mashburn and Whiteley,[39] membrane vesicles derived from *P. aeruginosa* cultures displayed potent anti-staphylococcal activity. This is because, in addition to PQS, these vesicles also contain large amounts of another AQ (again made by the PqsA-E-catalyzed pathway) called HQNO (2-heptyl-4-quinoline-N-oxide). HQNO strongly inhibits *S. aureus* growth.[28] PQS can also induce autolysis in *P. aeruginosa*, especially if overexpressed. In 2002, D'Argenio *et al.*[50] screened a Tn library to identify mutants with altered colony morphology. Several of these mutants exhibited pronounced autolysis (manifested as plaque-like clearings in the center of the colony) during growth on LB–agar plates. The same mutant colonies also had a metallic iridescent sheen; a phenotype well known to researchers familiar with many clinical isolates of *P. aeruginosa*. The two mutants with greatest autolysis contained independent Tn insertions in the gene encoding a monooxygenase, PqsL. These *pqsL* mutants overproduced PQS, and the autolysis phenotype could be alleviated through the introduction of secondary mutations in the *pqsA-E* biosynthetic cluster. The autolysis phenotype of the *pqsL* mutant could be mimicked by the addition of exogenous PQS. Not much is known about the function of PqsL, although speculatively it could be involved in the degradation or turnover of PQS. If so, and given its link with autolysis, PqsL could be an excellent target for antimicrobial intervention.

PQS signaling research continues to throw up surprises. In 2007, Farrow and Pesci[51] found that *phnAB*, long assumed to supply the main anthranilate precursor for PQS biosynthesis, was actually only transcribed during nutrient limitation. The majority of anthranilate used to supply PQS biosynthesis under nutrient-replete conditions comes from the breakdown of tryptophan, mediated by the kynurenine enzymes, KynABU. More recently, the same lab has shown that when a *P. aeruginosa* Trp auxotroph (i.e., a *trpE* mutant, unable to make its own tryptophan *de novo*) is plated on Trp-deficient media, bypass mutants that restore prototrophy (i.e., the ability to biosynthesize Trp *de novo*) arise with relatively high frequency.[52] Further analysis of these revertants revealed that they all contained the same mutation: G1041A in *pqsC*. This mutation gave rise to increased levels of *pqsD*, *pqsE*, and *phnAB* transcripts, and increased pyocyanin production (presumably, due to increased *pqsE* expression). Normally, the *pqsA-E* and *phnAB* genes are expressed on different transcripts. However, in the *pqsC* G1041A mutant, an alternative polycistronic transcript encompassing all four of the ORFs between *pqsD* and *phnB* is produced. The same transcript is produced under nutrient-limiting conditions (when *phnAB* are known to be required for PQS synthesis). It seems that inappropriate activation of *phnAB* expression can provide sufficient anthranilate not only to stimulate PQS-dependent pyocyanin production, but also to bypass the Trp auxotrophy of the original *trpE* mutant. We have devoted time to describe this work not only for its intellectual interest, but also because it provides a salutatory and very important lesson; whenever a pathway is inhibited, evolution inevitably finds a way to bypass the blockage. More recent data from Palmer *et al.*[53] indicated that overexpression of either *trpEG* or *phnAB* could compensate for PQS production or Trp auxotrophy in *trpEG* or *phnAB* mutants, which suggested that differential regulation of these genes played an im-

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portant role. Furthermore, these authors also showed that *trpEG* was expressed primarily during low-density growth, whereas *phnAB* was expressed primarily at high density.

In summary, PQS affects virulence in several distinct ways, some of which are direct and some are indirect. First, PQS induces expression of its own biosynthetic gene cluster (*pqsA-E*) in a PqsR-dependent fashion. Second, PQS-dependent PqsE expression impacts on the expression of many virulence genes, apparently through modulation of RhlR activity. Third, PqsR/PQS affects the expression of RsmA, which is a potent post-transcriptional regulator (whether this is linked with the effect(s) of PqsE on RhlR has yet to be investigated). Fourth, PQS affects the formation of extracellular trafficking vesicles. Finally, PQS also exhibits significant iron chelating capabilities, rapidly depleting the culture of iron by trapping it in an insoluble form. This iron trapping may have secondary effects, since iron depletion affects rhl signaling by enhancing *rhlR* transcription.

6 Open Questions

Over the last decade, much of the "the low hanging fruit" in PQS signaling research has been harvested, but a plethora of questions still remain. A selection of these (which unashamedly reflect the interests of these reviewers) include the following:

1) The PqsA-E/PhnAB pathway is now known to be responsible for synthesizing up to 50 different AQs. What are the function(s), if any, of these different AQs? Are some specifically tailored to mediate certain tasks? For example, there is evidence to suggest that the anti-staphylococcal HQNO plays little or no role in signaling, yet it is clearly potentially active in niche protection. Is the ratio of each AQ invariant, or is the synthesis of individual AQ species differentially regulated in some way? Is this plethora of AQs made simply because of biochemical "slop", or has promiscuity been evolutionarily hardwired into the biosynthetic enzymes?
2) Does anything degrade AQs? Most tightly regulated signaling pathways control the steady-state levels of the bioactive agent (signal) through a combination of regulated synthesis and degradation, so an AQ degradase would not be unexpected. Moreover, the AQ molecules represent a significant investment of metabolic resources, so one might expect some form of recycling, if only in the interests of frugality. Enzymes that degrade AHLs have already been identified in *P. aeruginosa* (e.g., PvdQ and QuiP). One possible *P. aeruginosa* PQS-degrading enzyme may be the monooxygenase encoded by *pqsL*; such an enzyme would be well-tailored for opening the anthranilate ring of the PQS molecule. Other bacteria, such as *Arthrobacter nitroguajacolicus* have been shown to encode an enzyme, Hod, which has 2,4-dioxygenase activity capable of digesting PQS,[54] although the activity of this enzyme against this substrate was low. Nevertheless, Hod was able to "quench" PQS-dependent QS to some extent.
3) How does PqsE synergize RhlR activity? Several scenarios present themselves (e.g., direct protein–protein interaction or the formation of a small molecule that allosterically activates RhlR), but, without further experimental insights, this problem may prove to be rather less tractable than it first appears. Resolution of the X-ray crystal structure of PqsE revealed that it had a metallo-β-lactamase fold with iron(II) or iron(III) at the active site (possibly substituted with Co^{2+} or Mn^{2+} *in vivo*), and that the protein cocrystallized with a benzoate ion (perhaps indicating a role in the enzymatic conversion of a chorismate-like species *in vivo*).[55] The purified enzyme could also slowly hydrolyze phosphodiesters and thioesters. However, no further functional insights could be gleaned from knowledge of the structure/enzymology alone. To begin to address this issue, Folch *et al.*[56] recently used alanine-scanning mutagenesis and molecular modeling to identify residues in the structure that were important for function. This approach revealed the importance of a C-terminal α-helical motif and showed that the presumed active site penetrated much deeper into the hydrophobic core of the protein than previously thought, but shed little further light on the mechanism by which PqsE exerted its activity. It seems then that PqsE is likely to have enzymatic activity, and that it is also likely to convert aromatic metabolites, but that remains the state-of-play for now.
4) How is the regulation of PQS-dependent virulence genes effected? PqsE clearly plays an important role as an effector of PQS signaling, but more needs to be done. For example, and as noted earlier in this review, in spite of many microarray analyses being carried out on PQS signaling, very few virulence genes, such as *lasB*, appear modulated in any of the studies. This is not to say that these genes are not modulated by PQS signaling, they certainly are (based on results obtained with reporter gene constructs and enzyme assays). However, there is a clear whiff of post-transcriptional regulation going on, and consistent with this, RsmA and another post-transcriptional regulator, Hfq, routinely appear to be modulated in most microarray analyses.
5) What is the role of MexGHI-OpmD in PQS signaling? Aside from the *pqsA-E* gene cluster, this quadruplet of genes are among the most highly modulated in all microarray analyses of PQS signaling, yet their precise role(s) remains unclear. The MexGHI-OpmD pump is unusual among *P. aeruginosa* RND-type

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multi-drug efflux pumps in that it is not strictly tripartite; the MexG protein is unique to this pump. Interestingly, MexG contains a predicted "DoxX"-like quinone binding domain.

6) If PQS signaling becomes blocked (as would be the case if this pathway were targeted for drug intervention), does the cell have any bypass mechanisms to circumvent the effect(s) of this? The data[21,52] (as well as previous experience) suggest that potential resistance mechanisms may indeed arise. The question is whether there will be sufficient selection pressure[22] to make this a problem (re: development of resistance to newly designed antimicrobial agents). We note that the costs of acquiring drug resistance can sometimes outweigh the benefits, especially when the selection pressure (drug) is removed.[57]
7) How is specificity achieved in the PqsR–PQS interaction? As discussed later on, the interaction between PQS and its receptor is almost exclusively driven by hydrophobic interactions, yet the bioactivity of the ligand is exquisitely sensitive to even minor structural modifications. This is a key issue, especially since the PqsR–PQS interaction is a key target for antimicrobial development.
8) PQS-stimulated membrane vesicles contain a very specific subset of secreted proteins compared with the bulk exoproteome. What determines which proteins become packaged? Can PQS derivatives be used to promote the "delivery" of vesicle-encapsulated compounds to bacterial cells?

7 A Job for the Chemists: PQS Signaling Pathways as a Potential Drug Target

In spite of its clear importance, PQS has been the subject of only very limited structure–activity relationship (SAR) analysis. Fletcher *et al.* compared the activities of PQS and HHQ analogues with different chain lengths on their ability to stimulate transcription from the *pqsA* and *lecA* promoters,[58] and more recently, Whiteley's group investigated how PQS and HHQ analogues with alkyl side chains containing seven, five, three, or zero carbon atoms influence MV production.[59] A more systematic SAR was initiated by Hodgkinson *et al.* in 2010.[60] These authors investigated the impact of alkyl chain length and ring modifications on PqsR-dependent transcription of *pqsA*, PqsR-independent stimulation of siderophore (pyoverdine) production, and membrane vesicle formation. Overall, peak stimulation of *pqsA* transcription by PqsR was achieved by PQS when the alkyl chain was seven carbon atoms long, although slightly longer-chain congeners retained significant activity (suggesting that the PqsR binding pocket can still accommodate these variants). However, replacement of the alkyl chain with a bulky aromatic substituent all but abolished binding, which indicates that the binding pocket is intolerant of non-alkyl moieties. Substitutions around the anthranilate ring had variable impacts: chlorine atoms generally rather uniformly reduced bioactivity (but did not abolish it), whereas introduction of a fluorine atom at position 5 had little effect, perhaps because it was much smaller than chlorine. In contrast, electron-donating substitutions in the ring, such as –OH or –OMe groups, had a much larger impact, and greatly reduced activity. Hodgkinson *et al.* also screened the same analogues for their ability to induce pyoverdine production. This phenotype was anticipated to be PqsR-independent, since it was presumed to arise as a result of the iron-trapping properties of PQS, leading to depletion of this element from the medium (and thereby stimulating siderophore synthesis). Surprisingly, the SAR profile of the PQS analogues in stimulating pyoverdine production was broadly similar to their profile in stimulating *pqsA* transcription (although the former assay had a much larger dynamic range). This is consistent with the findings of Rampioni *et al.*[46] who showed that siderophore synthesis was at least partially PqsE-dependent (recall that *pqsE* transcription is itself PqsR-dependent). Nevertheless, some portions of the AQ molecule clearly affected PqsR binding more than pyoverdine induction, and *vice versa*. Interestingly, none of the AQ analogues apparently had antagonistic activity. Indeed, and in contrast, Hodgkinson *et al.* noted that some physiologically relevant, but non-active AQ analogues strongly synergized the activity of PQS.[60] This raised the possibility that some of the "minor" AQs produced by PqsA-E might act to "fine-tune" PQS activity *in vivo*. This hypothesis remains to be tested. Finally, Hodgkinson *et al.* also examined how their analogues impacted on membrane vesicle formation. Unexpectedly, by far the most active compounds contained chlorine atom substitutions on the anthranilate ring, perhaps indicating that these substitutions facilitated the interaction between LPS and PQS. Overall, these researchers concluded that nearly every part of the AQ molecule made some contribution towards at least one of its known bioactivities. However, we have only just begun to scratch the surface in terms of detailed SAR, and much more needs to be done. Recent innovations with regards to the synthetic chemistry underpinning AQ analogue synthesis should help immensely here.[61,62]

Other recent SAR-related developments have included the introduction of quantitative approaches describing the binding of PQS to PqsR[63] and the long-awaited X-ray crystal structure of the PqsR ligand-binding domain (LBD).[64] The PqsR LBD contains Trp residues that can be excited by illumination at 292 nm and emit with $\lambda_{max} \approx 350$ nm. Fortuitously, PQS and other AHQs absorb strongly at 350 nm and, if excited at this wavelength, emit at around 450 nm. This overlap of Trp emission and PQS excitation spectra immediately suggested to Sams and colleagues that nonradiative resonance energy transfer

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(RET) might occur if the ligand was bound close to one of the Trp fluors.[63] Indeed, a mixture of the PqsR LBD and PQS excited at 292 nm only showed pronounced emission at >400 nm. This 'textbook' example Förster resonance energy transfer (FRET) was used by this team to quantify PQS binding to PqsR. They used these data to derive a quantitative model of ligand binding, in which two PQS molecules bound consecutively to each dimer of PqsR with a K_d of about 1.2 μM. Consistent with this, Ilangovan *et al.*[64] noted that the LBD of PqsR was dimeric in the crystal structure. Each monomer in the dimer is comprised of two domains, and the monomers are arranged in an antiparallel fashion with respect to one another. One of the domains in each monomer contains a hydrophobic pocket, lined by Ile, Leu, and Val residues. The relatively close juxtaposition of two such pockets (one from each monomer in the dimer) creates a larger, *trans*-dimer hydrophobic cavity, which is enclosed by loop structures. Crystal soaking experiments revealed that AQs were most likely to bind within these hydrophobic pockets. Remarkably, it appears that AQs are likely to be bound exclusively through hydrophobic interactions (which would explain why PQS binding to the PqsR LBD cannot be monitored using calorimetric approaches (M.W., J.G., unpublished data)). Consistent with this, mutation of the hydrophobic residues lining the AQ binding cavity led to reduced bioactivity. Clearly, this leaves open the question of how specificity is achieved in binding; presumably, steric effects have a role to play, as may interactions with selected hydrophilic residues or π systems (Phe side chains) in the vicinity of the AQ binding pocket. Indeed, in the same study,[64] Ilangovan *et al.* reported that a PQS analogue (7-Cl-PQS), containing a chlorine atom at position 7 on the ring, behaved as a "super-agonist", displaying 135-fold greater potency than PQS itself. Analysis of a similar molecule bound to the LBD of PqsR revealed that a chlorine atom at this position could form a hydrogen bond with the side chain of Thr265. Clearly, there is potential for "directed" (enthalpy-driven) interactions here, and this may need to be exploited in drug development programs.

8 Blocking AQ-Dependent QS

Clearly, if PQS-dependent QS can be disrupted, this may have therapeutic potential. The virulence of the organism will be suppressed, as will its ability to form biofilms, making it more sensitive to killing by the host immune system and "conventional" antibiotics. An oft-debated question is whether such compounds will elicit a "weaker" selection pressure (whatever that means) with respect to the evolution of resistance, and thereby exhibit greater "shelf longevity". This issue still needs to be explicitly experimentally addressed. However, it has not stopped many groups (including ours) from taking up the mantle and trying to identify antagonists that block PQS signaling. Several targets present opportunities in this regard, including PQS binding to PqsR (MvfR), inhibition of PQS synthesis, activation of *pqsL* transcription and/or activity, or disruption of PqsE activity. The first two options present the path of least resistance, since more is known about the biology underpinning these.

Two early studies from 2007 (Cugini *et al.*[65] and Lesic *et al.*[66]) identified inhibitors of PqsR-dependent signaling and PQS biosynthesis, respectively. Cugini *et al.* found that the sesquiterpene, farnesol, produced by the fungus *Candida albicans*, bound to PqsR, promoting the formation of a nonproductive complex between PqsR and the *pqsA* promoter. Indeed, addition of either purified farnesol to PA14 cultures or coculture with *C. albicans* lead to a pronounced diminution in pyocyanin production. More recent work by the same team has shown that, under some circumstances, farnesol can also *stimulate* PQS production by promoting *pqsH* transcription.[67] Indeed, farnesol was able to overcome the pyocyanin and PQS defects in a *lasR* mutant. This stimulation of *pqsH* expression was dependent upon RhlR. The effects of farnesol in restoring PQS signaling in a *lasR* mutant could be abrogated by addition of a reducing agent, *N*-acetylcysteine (NAC), and mimicked by addition of H_2O_2. This, coupled with the fact that farnesol is known to induce the production of reactive oxygen species (ROS), strongly suggests that ROS may promote rhl signaling. Interestingly, NAC apparently has beneficial effects in CF patients. This was originally thought to be due to its ability to suppress ROS accumulation, although the work of Cugini *et al.* suggested that it might also affect QS. Lesic *et al.* adopted a different "quorum quenching" strategy.[66] They identified a selection of three halogenated anthranilate analogues (2-amino-6-fluorobenzoate (6FABA), 2-amino-6-chlorobenzoate (6CABA), and 2-amino-4-chlorobenzoate (4CABA)) that blocked the synthesis of AQs by PqsA-E. The most likely target of these competitive inhibitors was the CoA-ligase, PqsA, which was involved in activating aromatic carboxylates (such as anthranilic acid); the earliest step in PQS biosynthesis. The 6FABA, 6CABA, and 4CABA compounds did not affect bacterial growth, but very effectively blocked HHQ/PQS synthesis and the expression of MvfR-dependent virulence genes. They also increased host survival during *P. aeruginosa* infection (mouse model) and restricted *P. aeruginosa* dissemination during infection.

Other groups have focused their attention on identifying antagonists of PqsR. Lu and colleagues reported the first PqsR antagonists in early 2012.[68] They modified the HHQ core structure. Although HHQ is a much less potent ligand compared with PQS, it does not chelate iron and does not promote membrane vesicle formation, which simplifies interpretation of the results. Their analysis showed that HHQ analogues containing strong electron-withdrawing substituents at the 6-position on the

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ring were antagonistic, with greater antagonistic potency correlating with the electronegativity of substitution. In their next publication, the team went one step further by using a combination of rational design and surface plasmon resonance (SPR)-based binding assays to further refine antagonist activity.[69] However, they did not use their previously identified HHQ analogue(s) as a starting point. Instead, they noted that recent work by Zaborina *et al.*[70] showed that the κ opioid receptor agonist, (±)-*trans*-U50488, strongly stimulated *pqsA-E* expression in *P. aeruginosa*, presumably by binding to PqsR. They therefore chose to use this as a starting scaffold to identify potent antagonists of PqsR. A library of derivatives was made and screened by SPR to identify PqsR binders. Four "hits" were identified, and binding of the best of these (4-chlorobenzamide (4CB)) to PqsR was quantified by ITC (K_d = 25 μM). This raises an important point, also alluded to earlier: although PQS binding appears to be largely driven by hydrophobic interactions (which are "invisible" to isothermal titration calorimetry (ITC)), opportunities do exist within the ligand binding pocket for more directed, enthalpically driven binding interactions. 4CB was found to be an antagonist of PqsR, so further derivatives were made and one, *tert*-butyl benzamide, displayed even tighter binding to PqsR (K_d = 0.9 μM). However, *tert*-butyl substitution resulted in partially restored agonist activity, so additional modifications were made to suppress this. Eventually, it was found that, when the amide in *tert*-butyl benzamide was replaced with a hydroxamate moiety, full antagonism was restored with moderately good binding affinity to PqsR (K_d = 4.1 μM). Moreover, this lead compound retained weak antagonistic activity in vivo (IC_{50} for pyocyanin production was 87 μM), which was not bad when considering that many xenobiotics were readily expelled from this organism by its plethora of multi-drug efflux pumps. The Williams team have also been exploiting their structural data on the $PqsR_{LBD}$ to also identify PqsR antagonists.[64] Their starting template was the 2-alkyl-4(3*H*)-quinazolinone (QZN) system with C7 or C9 alkyl side chains. A library of these compounds with varying substitutions was screened for antagonistic activity. Importantly, this screening was done in *P. aeruginosa*. One of the compounds, 3-NH_2-7Cl-C9-QZN (Figure 3), exhibited potent antagonist activity (IC_{50} 5 μM) in a *pqsA* transcription assay. Similar to the superagonist (7-Cl-PQS) identified in the same study, 3-NH_2-7Cl-C9-QZN contains a chlorine atom at position 7,

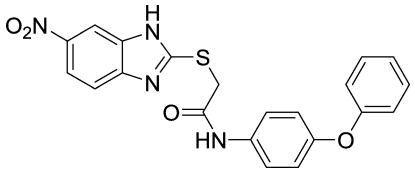

Figure 3. Chemical structure of M64; a potent inhibitor of MvfR.[71]

which makes contact with the side chain of Thr265, promoting binding. It is reassuring that 3-NH_2-7Cl-C9-QZN was also able to suppress pyocyanin, biofilm, and virulence factor transcription in *P. aeruginosa*, as well as PQS production, although, in most cases, high concentrations of this competitive antagonist were required. However, perhaps the most effective efforts at blocking MvfR-mediated QS have once again been made by Rahme's team.[71] These researchers developed a simple but elegant genetic screen, in which the expression of *sacB* was driven by the *pqsA* promoter. SacB is a levansucrase, which, in the presence of sucrose, generates a toxic product that kills the cell. Therefore, cell growth will only be seen if transcription from the *pqsA* promoter is shut down. Compounds that inhibit MvfR should do this. A screen of >284000 compounds yielded 39 hits, 8 of which shared a common scaffold: the benzamide–benzimidazole backbone. All of these compounds were effective at <10 μM, and one of the more potent (M64) was shown to bind directly to purified MvfR with a K_d of 5.4 nM. Since M64 (Figure 4) does not structurally resemble PQS or HHQ, there is hope that this compound might not be

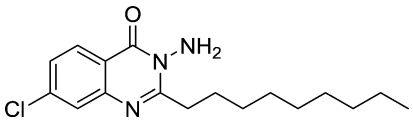

Figure 4. Chemical structure of 3-NH_2-7Cl-C9-QZN; a potent antagonist of PqsR.[64]

a competitive inhibitor, although this has yet to be tested. Presumably, structural and detailed kinetic analyses will establish the precise mechanism of action. M64 was active against multi-drug-resistant isolates and suppressed both acute and persistent murine infections without perturbing bacterial growth. In addition, it also reduced the formation of antibiotic-tolerant persister cells; all factors that bode very well indeed.

9 Outlook

Over the last 15 years, PQS research has gone from strength-to-strength, and as is the case with its older sibling (AHL-dependent QS), surprises are still being thrown up with alarming regularity; this is far from being a "solved" field of research. Just a few of the outstanding questions have been highlighted herein, and we apologize to colleagues whose work has not been cited for reasons of space limitation. The potential of this system as a target for antimicrobial development is being realized, although some caution is still required as to the likely clinical utility (in terms of resistance arising) of strategies that target nominally "nonessential" physiologies, such as virulence. For obvious reasons, most current work in this

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area has focused on the development of *competitive* inhibitors of PQS signaling or PQS synthesis. However, in the longer term, it is clear that we will need to identify noncompetitive or uncompetitive blockers; this is a much taller order. Additional structural and biochemical data will surely help, as will a better understanding of the mechanism(s) underpinning some of the more elusive potential targets, such as PqsE.

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