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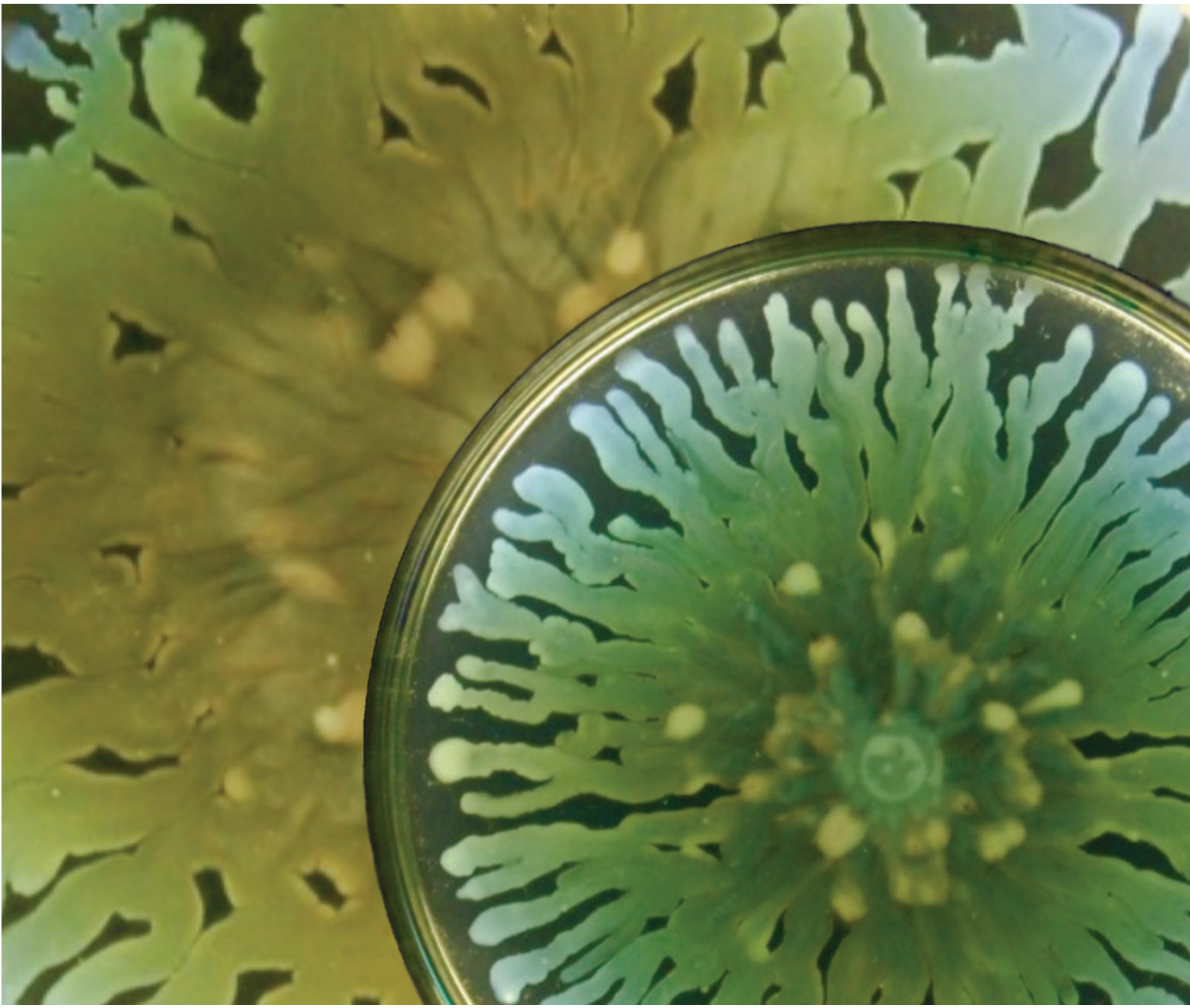

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Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species

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Pseudomonas aeruginosa produces the cell-to-cell signal molecule 2-heptyl-3-hydroxy-4-quinolone (The *Pseudomonas* quinolone signal; PQS), which is integrated within a complicated quorum sensing signaling system. PQS belongs to the family of 2-alkyl-4-quinolones (AQs), which have been previously described for their antimicrobial activities. PQS is synthesized *via* the *pqsABCDE* operon which is responsible for generating multiple AQs including 2-heptyl-4-quinolone (HHQ), the immediate PQS precursor. In addition, PQS signaling plays an important role in *P. aeruginosa* pathogenesis because it regulates the production of diverse virulence factors including elastase, pyocyanin and LecA lectin in addition to affecting biofilm formation. Here, we summarize the most recent findings on the biosynthesis and regulation of PQS and other AQs including the discovery of AQs in other bacterial species.

1. Introduction

Until relatively recently, bacteria were considered autonomous single-celled organisms with little capacity for collective behaviours. However, we now appreciate that bacterial cells are highly communicative and possess an extraordinary capacity for social behaviors. Bacteria coordinate their activities by producing and detecting small diffusible signal molecules which enables a population of bacteria to behave collectively rather than as individuals. Such cooperative behaviour is known as "quorum sensing" (QS) and plays a pivotal role in the lifestyles of both beneficial and pathogenic bacteria. *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium that is capable of surviving in a broad range of natural environments, although it is best known as an antibiotic resistant human pathogen associated with hospital acquired infections[1,2] and as a leading cause of death in cystic fibrosis (CF) patients. In *P. aeruginosa*, cell–cell communication is known to control the production of extracellular virulence factors and promote biofilm maturation.[3–5] The QS system consists of two *N*-acylhomoserine lactone (AHL) regulatory circuits (*las* and *rhl*) linked to a 2-alkyl-4-quinolone (AQ) system. In the *las* system, the *lasI* gene product directs the synthesis of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which interacts with the transcriptional regulator LasR and activates target promoters. In the *rhl* system, *rhlI* directs the synthesis of *N*-(butanoyl)-L-homoserine lactone (C4-HSL), which interacts with the cognate regulator RhlR and activates target gene promoters. The *las* and *rhl* systems are hierarchically connected and have been found to regulate the timing and production of multiple virulence factors including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, superoxide dismutases and biofilm formation.[6]

Pesci *et al.* (1999) demonstrated that addition of a spent culture medium extract from a *P. aeruginosa* wild type (PAO1) caused induction of *lasB* (codes for elastase) expression in a PAO1 AHL deficient *lasR* mutant.[7] This data suggested that a non-AHL signal produced by the bacterium was capable of activating *lasB* expression and which required LasR and 3O-C12-HSL for its biosynthesis. It was also shown that the novel signal required a functional *rhl* system for its bioactivity since *lasB* could not

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Dr Jean-Frédéric Dubern studied biochemistry at the University of Montpellier II, France. He received his PhD in molecular microbiology in 2006 from Leiden University, The Netherlands. His research focused on the role and genetic regulation of cyclic lipodepsipeptide biosynthesis in plant beneficial rhizobacteria. Since 2006 he has been a Marie Curie Research Fellow at the Institute of Infection, Immunity & Inflammation at the University of Nottingham, United Kingdom. His current research activity is focused on the metabolic consequences of quorum sensing in bacteria.

Dr Steve Diggle has studied the role of quorum sensing on the virulence of Pseudomonas aeruginosa in the group of Prof. Paul Williams (University of Nottingham) since 1997. After completing his PhD in 2001, his post-doctoral work focused mainly on the role of 2-alkyl-4-quinolones (AQs) in P. aeruginosa. In 2006, he received a fellowship from the Royal Society to study social behaviours in pathogenic bacteria. Much of his work now focuses on understanding how bacterial communication systems evolve and are maintained in natural environments. His work can be viewed at http://www.nottingham.ac.uk/quorum/diggle.htm.

be activated in a *rhlI*/*rhlR* double mutant by PAO1 spent culture extracts.[7] The molecule responsible for the non-AHL-mediated QS signalling pathway was purified and chemically identified as 2-heptyl-3-hydroxy-4-quinolone and termed the Pseudomonas Quinolone Signal (PQS). PQS belongs to the AQ family of compounds which were first chemically identified in the 1940s and studied for their antibacterial properties.[8] In addition to PQS, other major molecules produced by this organism which belong to this family include 2-heptyl-4-quinolone (HHQ), 2-nonyl-4-quinolone (NHQ) and 2-heptyl-4-quinolone *N*-oxide (HHQNO).

2. Biosynthesis of 2-alkyl-4-quinolones (AQs)

Studies on the synthesis of AQs show that they are derived *via* the condensation of anthranilate and a β-keto-fatty acid.[9] It was demonstrated that anthranilate is a precursor for PQS and that addition of an anthranilate analogue, methyl anthranilate (which inhibits anthranilate synthetases) to a *P. aeruginosa* culture disrupts PQS production.[10] In addition, Bredenbruch *et al.* (2005) showed that the C_4 of PQS derives from anthranilic acid and C_2 derives from acetate, thereby proving that anthranilate and a fatty acid combine to produce 2-heptyl-4-quinolone (HHQ).[11]

Interestingly, the genome of *P. aeruginosa* encodes multiple proteins that are similar to anthranilate synthases, but only two of these (TrpEG and PhnAB) appear to supply anthranilate that is available for general cellular functions.[12] The anthranilate synthases are encoded by *phnAB* located adjacent to the *pqsABCDE* operon and these were shown to be part of the main metabolic pathway that provides anthranilate for PQS biosynthesis since *phnAB* is co-regulated with *pqsABCDE* operon.[13,14] In addition, the three genes (*kynA*, *kynB*, and *kynU*) of the anthranilate branch of the kynurenine (*kyn*) pathway, which convert tryptophan to anthranilate, are present in *P. aeruginosa*.[15] The importance of this catabolic pathway for PQS biosynthesis was suggested by D'Argenio *et al.* (2002).[16] Recent experiments from Farrow *et al.* (2007) showed that supplementing a *P. aeruginosa* culture with radioactive tryptophan resulted in the production of a radio-labeled PQS and that kynurenine pathway mutants could not produce radioactive PQS.[15] The kynurenine pathway was proposed to function *via* three enzymes, in which tryptophan would be converted to formyl-kynurenine by a tryptophan 2,3-dioxygenase (KynA), formyl-kynurenine converted to kynurenine by a kynurenine formamidase (KynB), and kynurenine to anthranilate by a kynureninase (KynU).[15] In their study, Farrow *et al.* (2007) showed that *kynA* and *kynU* mutants produced no PQS and a *kynB* mutant produced a reduced amount of PQS providing evidence that an alternative pathway to *phn* may supply anthranilate.[15] However, a kynurenine pathway mutant continued to make PQS in minimal medium. Under these conditions, a *phnA* mutant did not produce PQS and PQS synthesis is restored by addition of either exogenous anthranilic acid or providing with *phnAB* genes in trans. Thus, further studies of the regulation and activities of *kyn* and *phn* pathways are necessary to determine the specific environmental conditions which cause them to become active (Fig. 1).[15] The presence of two independent pathways leading to anthranilate synthesis, which is the direct precursor in AQ biosynthesis in *P. aeruginosa* implies that anthranilate is an important metabolite for the pathogenesis of *P. aeruginosa*.

The gene cluster which directs the biosynthesis of AQs in *P. aeruginosa*, was identified during a random transposon mutagenesis screen for regulators of pyocyanin production and termed *pqsABCDE*.[13] PqsA was identified as a probable benzoate coenzyme A ligase involved in anthranilate activation, while PqsB, PqsC and PqsD show similarities

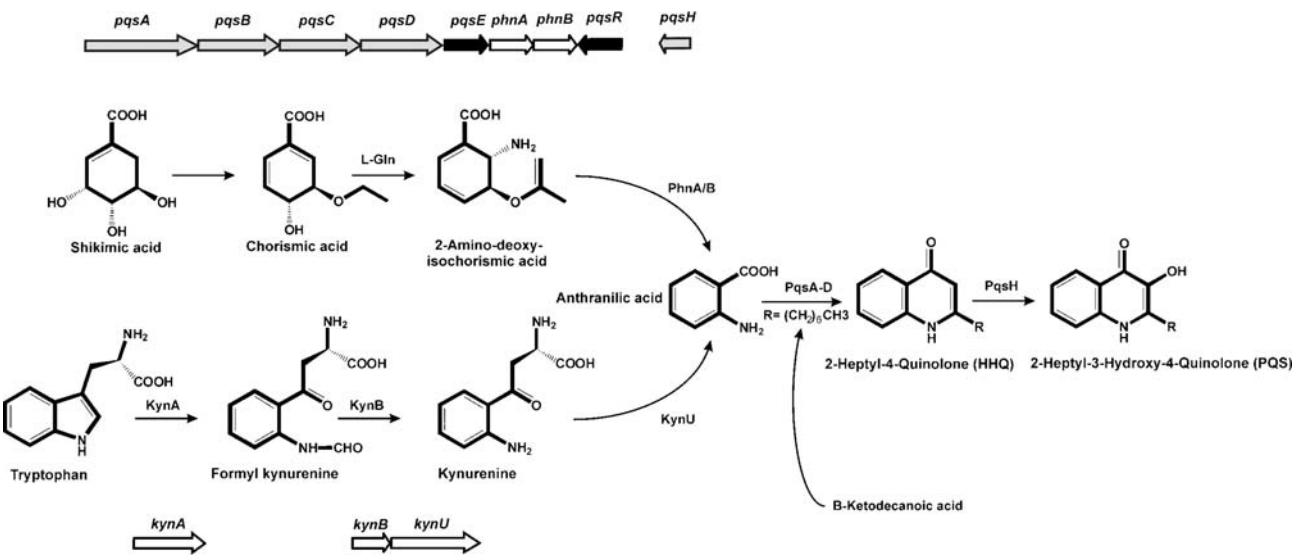

Fig. 1 The PQS biosynthesis of *P. aeruginosa*. Two distinct metabolic routes provide anthranilate for PQS synthesis by either PhnAB anthranilate synthetase from shikimic acid *via* chorismic acid or tryptophan degradation depending on environmental conditions. The *pqsABCDE* operon is regulated by the LysR-family regulator PqsR. Anthranilic acid condenses with β-ketodecanoic acid to produce 2-heptyl-4-quinolone (HHQ). The FAD-dependant monooxygenase PqsH converts PQS precursor HHQ into PQS.

with β-keto-acyl-acyl carrier protein synthetases. Anthranilate is converted by the *pqsABCD* gene products into HHQ (2-heptyl-4-quinolone), the precursor of PQS.[14] HHQ can be passed between cells and converted intracellularly into PQS through the action of PqsH, a probable FAD-dependent monoxygenase (Fig. 1).[13,14] The transcription of the *pqsH* gene was shown to be positively controlled by LasR, providing a link between the AHL and AQ quorum sensing systems.[13] Along with PQS and HHQ, *P. aeruginosa* produces at least 55 quinolone compounds, many of which are produced by the action of the *pqsABCD* genes and some of which have been chemically identified because of their antibiotic activities.[17,18]

Two other genes, *pqsL* and *pqsE*, were identified and suggested to play a role in PQS biosynthesis. A mutation in *pqsL* resulted in an overproduction of PQS.[16] Interestingly, Lepine *et al.* found by profiling the production of AQs of *P. aeruginosa* that no AQ *N*-oxides were detected in a *pqsL* mutant, indicating that this gene is involved in the biosynthesis of these particular AQ compounds. The role of PqsE, a putative metallo-β-lactamase, is largely unknown. A *pqsE* mutant does not produce pyocyanin or PA-IL lectin (LecA) but produces wild type levels of PQS and HHQ, suggesting that PqsE could possibly be required for the cellular response to PQS by generating an as yet unidentified signalling molecule from PQS.[13,19]

3. The regulation of AQs and their role in cell-to-cell communication

AQs including PQS can be detected during the logarithmic phase of growth.[19,20] The regulation of the AQ biosynthetic genes including the *phnAB* and *pqsABCDE* operons, occurs through a LysR family transcriptional regulator called PqsR (MvfR),[13,14] which is positively controlled by LasR/3-oxo-C12-HSL.[21] PqsR was predicted to be membrane associated and its discovery was made during the search for genes which regulate pyocyanin production when it was demonstrated that a *pqsR* mutant was unable to produce either pyocyanin or PQS.[13,22] PqsR binds the promoter of *pqsABCDE* but this binding increases dramatically in the presence of PQS, suggesting that PQS acts as a PqsR co-inducer.[21,23] The *pqsABCD* gene products direct the synthesis of HHQ and originally this molecule was suggested to be an extracellular messenger, released by one cell, taken up by a neighboring cell and converted into PQS by the action of PqsH.[14] However, PQS does not work alone in activating *pqsABCDE*. It is now known that HHQ can also potentiate PqsR binding to the *pqsABCDE* promoter (Fig. 2).[23] Not only that, PQS is fully dispensable, as a *pqsH* mutant which produces HHQ but no PQS displayed normal PqsR-dependent gene expression and virulence.[23] This demonstrates that HHQ can also function as a signal molecule per se and shows that there is still work to do to fully understand the mechanisms as to how AQs regulate gene expression.[23,24]

It has also been established that PQS regulates the RhlI/R system. The first indication that PQS was interconnected with the *rhl* system was that the bioactivity of PQS (measured through the ability of PQS to activate a *lasB–lacZ* fusion in late stationary phase) required a functional RhlR gene, suggesting that PQS was acting through the *rhl* QS system.[7] Later, it was shown by McKnight *et al.* (2000) that PQS positively regulates *rhlI* expression, thus influencing C4-HSL accumulation.[25] Recently, it was observed that *pqsABCDE* expression depends on the ratio between the two AHL molecules, 3-oxo-C12-HSL having a positive effect, and C4-HSL having a negative effect[26] and that *pqsR* was a target for a negative regulation by *rhl* system.[22] Thus *las* and *rhl* systems act antagonistically on *pqsR* regulation (Fig. 2). However, it is important to note that under certain growth conditions there can be considerable LasR-independent synthesis of PQS and so *P. aeruginosa* is adaptable at producing AQs.[19] This could be especially important in environments such as the cystic fibrosis lung where *lasR* mutants are often isolated and yet AQs can still be isolated.[27,28]

Given that PQS can act as a cell-to-cell signalling molecule, how then is it released from a cell? Firstly it is important to note that the activity of a cell-to-cell signaling molecule depends on its ability to dissolve and diffuse freely through aqueous solutions. PQS is relatively insoluble in aqueous solutions, suggesting that *P. aeruginosa* produces a PQS-solubilizing factor. It was shown that the biosurfactant rhamnolipid produced by *P. aeruginosa* greatly increases solubility of PQS in aqueous solution, resulting in an increase in PQS bioactivity.[29] A mechanism for PQS release has been proposed as it has been demonstrated that *P. aeruginosa* packages the molecule into membrane vesicles that serve to traffic PQS within a population.[30] TLC analysis showed that a large amount of PQS was present within membrane vesicles (MVs).[30] LC-MS/MS analysis also showed that the MVs contained HHQ and HNQ.[30] These findings illustrate that *P. aeruginosa* could use a signal trafficking system with features common to those used by higher organisms for the delivery of a signal critical for coordinating group behaviour.

Whilst the molecular mechanisms governing AQ production in *P. aeruginosa* are being unravelled, it is now known that the environmental conditions also play a role in the levels of AQs produced by *P. aeruginosa*. The role of phosphate limitation on PQS and pyocyanin production was recently established by Jensen *et al.* (2006).[31] AQ production was enhanced under low-phosphate medium conditions, and the induced AQ production was abolished in a *phoB* mutant.[31] Since in a *phoB* mutant *rhlR* expression was induced under iron-limited conditions, the study examined whether PQS could activate *rhlR* expression in a *phoB*-independent manner. PQS addition to the wild type and the *phoB* mutant increased *rhlR* expression under high-phosphate conditions. However, although PQS activates *rhlR* transcription in a PhoB-dependant manner, a *phoB* mutant could not be stimulated by PQS to produce a high level of pyocyanin, suggesting that pyocyanin production is under the strong influence of PhoB in addition to the effect of PQS. These results suggest that PhoB links environmental conditions and cell density-dependent systems to secondary metabolite production in *P. aeruginosa*.

4. The biological functions of AQs in *P. aeruginosa*

Both PQS and HHQ have both been shown to play a role in cell-to-cell communication, however recent studies have shown that PQS appears to have a number of other biologically important functions (Fig. 3).

Two recent studies[24,32] have demonstrated that PQS influences *P. aeruginosa*

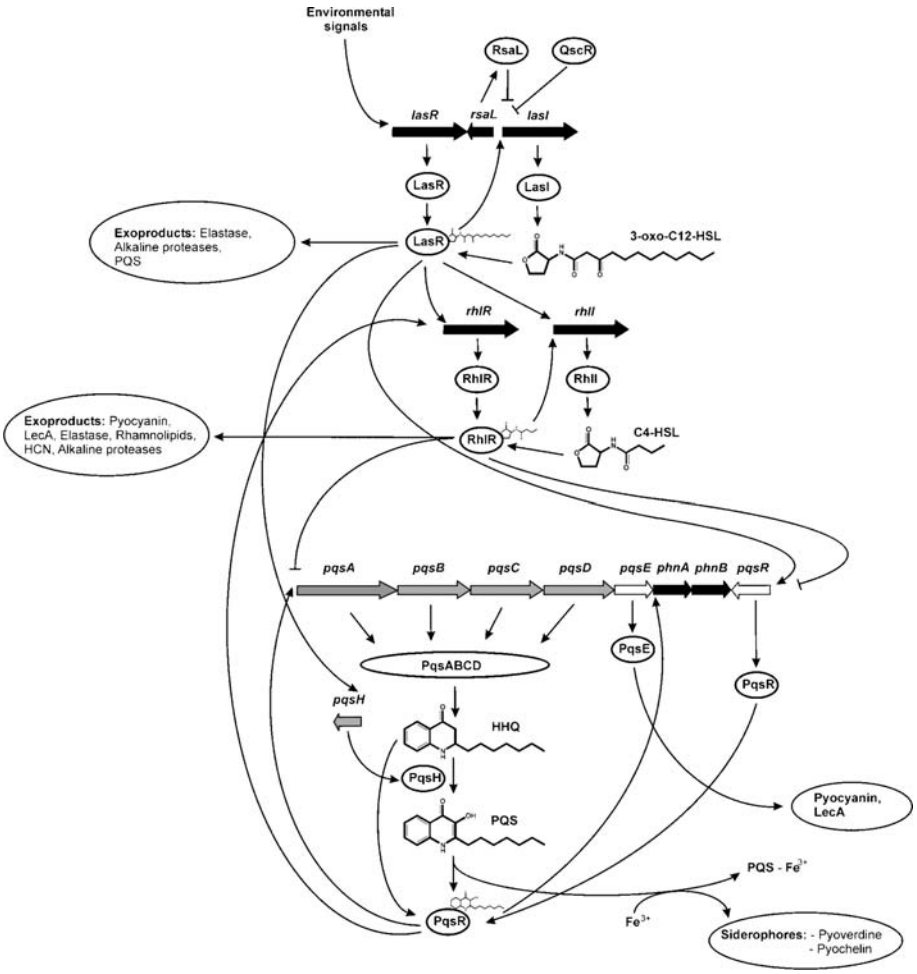

Fig. 2 *P. aeruginosa* cell-to-cell signaling. AHQ and AHL-dependant quorum sensing are intimately linked since LasR/3-oxo-C12-HSL is required for full expression of *pqsH*, while *pqsR* is positively regulated by LasR/3-oxo-C12-HSL. Both *pqsA* and *pqsR* are repressed by the action of RhlR/C4-HSL system. HHQ is produced *via pqsA* itself regulated by *pqsR*. Both PQS and HHQ induce the expression of *pqsA* in a PqsR-dependant manner. The production of lectin and pyocyanin also require PqsE. Furthermore, PQS released from the cell is capable of binding iron, forming a PQS-Fe^{3+} complex. The removal of iron from the extra-cellular environment by PQS induces expression of genes involved in siderophores production in an independent manner from cell-to-cell signalling. → represent positive regulation; ⊣ represent negative regulation.

iron homeostasis and this is an important step in the understanding of complex bacterial interactions as there is growing evidence that cell-to-cell signaling mediated by QS systems can also be strongly affected by environmental factors other than cell density. Bredenbruch *et al*. (2006) performed a transcriptome analysis of PAO1 cultures supplemented with PQS. The transcriptome profile revealed an induction of the iron acquisition systems as well as the oxidative stress response upon PQS supplementation.[32] In addition, when PQS was added to a culture, a rapid loss of free iron was observed. This was confirmed by ESI/MS analysis showing that PQS chelates iron in a 3 : 1 complex.[32] More recently, a biophysical analysis conducted by Diggle *et al*. (2007) revealed that PQS forms a complex with iron(III) at physiological pH (7.4).[24] However, PQS does not appear to act as a siderophore but rather as an iron trap. When PQS was supplied to a *P. aeruginosa* mutant unable to produce pyoverdine or pyochelin, PQS associated with the cell envelope and inhibited bacterial growth, suggesting that PQS could play a role in iron entrapment to facilitate siderophore-

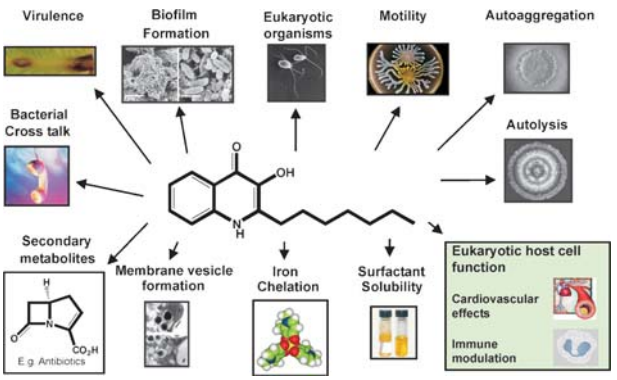

Fig. 3 Biological roles of PQS and phenotypes known to be regulated by PQS in *P. aeruginosa*.

mediated iron delivery.[24] In natural environments where competition for iron is important, PQS may aid *P. aeruginosa* growth by trapping iron and storing it in the cell membrane for future use. Not only that, PQS-mediated iron chelation may also starve competing species of bacteria of free iron in the environment.

PQS has also been shown to be involved in biofilm formation. Addition of PQS to cultures of *P. aeruginosa* PAO1 resulted in enhanced attachment to stainless steel coupons.[19] The mechanism for this was not described but one possibility may be PQS-induced production of the galactophilic lectin LecA which has been shown to be important for the full maturation of biofilms.[19,33] A second possible mechanism is the release of extracellular DNA which is one of the major matrix components in *P. aeruginosa* biofilms. Evidence has been presented that QS plays a role in the formation of extracellular DNA in *P. aeruginosa* biofilms.[34] QS-regulated DNA release from *P. aeruginosa* strains involves lysis of a sub-population of cells.[34] Biofilms formed by a *pqsA* mutant contained less extracellular DNA than biofilms formed by the wild type. Not only that, the mutant biofilms were less susceptible to treatment with sodium dodecyl sulfate than a wild type biofilm.[34] In support of this, *P. aeruginosa* strains showing a high level of autolysis overproduced PQS.[16] This increase in PQS may be a mechanism for the release of extracellular DNA which results ultimately in an increase in biofilm development.[19,34]

Interestingly there appears to be a link between iron levels, DNA release and biofilm formation. It has been shown that *pqs* gene expression, DNA release and biofilm formation were favoured in media with low iron concentrations. When levels of iron were high, it was found that *pqsA* expression was significantly lower, DNA release was repressed and biofilms were structurally altered resulting in biofilms with a higher susceptibility to antimicrobial compounds.[35] Expression studies showed that the *pqs* operon was induced in particular sub-populations of biofilm cells under low iron conditions and repressed in biofilm cells when higher iron level was tested.[35] Therefore, PQS could be involved in a fine balance between survival and persistence of bacterial cells in the environment.[16]

A recent and interesting paper looked at the role of the HHQNO molecule. It has long been known that HHQNO has properties that make it effective against *Staphylococcus aureus*. This recent study showed that HHQNO suppresses *S. aureus* respiration.[36] Presumably this gives *P. aeruginosa* an ecological advantage when both species are growing together in an environment such as the cystic fibrosis lung. However, it was shown that HHQNO resulted in the development of *S. aureus* small colony variants (SCVs) which show stable amino-glycoside resistance.[36] Therefore, the production of HHQNO may in fact also be advantageous to *S. aureus* due to increased resistance to certain classes of antibiotics.

There is little still known about the roles of these molecules within a host. What is important to note is that these molecules can be detected directly from infected hosts. *P. aeruginosa* is a major cause of morbidity and mortality in cystic fibrosis (CF) patients.[37] PQS has been detected in sputum, bronchoalveolar lavage fluid and mucopurulent fluid from *P. aeruginosa* infected CF patients.[28] The levels of PQS estimated to be present correlated with the population density of *P. aeruginosa* in the sample.[28] Adaptation to the CF airway occurs during the first 3 years of life and interestingly *P. aeruginosa* strains isolated from 24–36 month old CF patients produced 7–15 fold more PQS than the laboratory strain PAO1 during logarithmic growth. However, in isolates from patients older than 36 months, PQS biosynthesis was significantly reduced suggesting that PQS production may vary during the different stages occurring during the establishment of *P. aeruginosa* in the CF lung.[38]

The interactions between quorum sensing signals and the host immune response has also been explored. In early immunological experiments, 3-oxo-C12-HSL was shown to suppress interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) secretion by LPS-stimulated macrophages and to eliminate T-cell proliferation.[39] In recent studies Hooi *et al.* (2004) determined that the signal molecule PQS was also capable of modulating the immune response in a manner similar to that described for 3-oxo-C12-HSL.[40] In human peripheral blood mononuclear cells (hPBMC) activated with a lectin, both signals showed immune suppression in terms of cell proliferation and cytokine (IL-2) release. However, following LPS activation of monocytes in hPBMC, the release of TNF-α was unaffected by treatment with PQS provided at relatively low concentration (10 μM) when compared with 3-oxo-C12-HSL. These results offer the possibility of developing quorum sensing signals for therapeutic purposes as novel immune modulators. There are very few reports describing what influence signals produced by a host have on the cell-to-cell communication systems in bacteria. Interestingly, dynorphin, a K-opioid which is released from intestinal mucosa following ischemia in mice, activates AQ signaling in *P. aeruginosa*.[41] Dynorphin was demonstrated to stimulate expression of *pqsABCDE*, production of HHQNO, HHQ, and PQS, and in turn enhance virulence factor production including pyocyanin and LecA, suggesting that *P. aeruginosa* can respond to opioid compounds released during host stress by regulating key elements of the quorum sensing system resulting in enhanced virulence.[41]

5. 2-Alkyl-4-quinolone signaling in other bacteria

Since AQs such as PQS are derived from fatty acid biosynthesis and anthranilate being supplied *via* a common tryptophan biosynthesis pathway, other bacterial species may be capable of synthesizing similar molecules. Interestingly, Diggle *et al.* (2006) showed that the genome of at least two *Burkholderia* species, *Burkholderia pseudomallei* and *Burkholderia thailandensis*, possess homologous operons to the *pqsABCDE* biosynthetic gene cluster and these were termed *hhqABCDE*.[42] Complementation of a *P. aeruginosa pqsA* mutant with the *hhqA* gene from *B. pseudomallei* completely restored production of HHQ and PQS. Not only that, complementation of a *P. aeruginosa pqsE* mutant could be achieved by the *hhqE* gene. This indicates that the operons in *B. pseudomallei* and *B. thailandensis* are fully functional. In addition, LC-MS/MS analysis confirmed that *B. pseudomallei*, *Burkholderia cenocepacia*, as well as *Pseudomonas putida* produce AQs including HHQ.[42] A more recent study

identified a new metabolite, 2,4-dihydroxyquinoline (DHQ) in both *P. aeruginosa* and *B. thailandensis*, the production of which is governed by PqsA/HhqA.[43] DHQ is neither a degradation product or precursor of AQs, nor does it increase the expression of *pqsA* or pyocyanin production.[43] Its major function, like for many of these compounds remains a mystery. The discovery of AQs in *Burkholderia*, a genus distinct from the fluorescent pseudomonads, indicates that AQ-dependent signalling may be more widespread than previously thought and offers the exciting possibility that AQs may be involved in both inter-genus and inter-species signalling.

6. Conclusions and future perspectives

Our understanding of the molecular mechanisms governing AQ production in *P. aeruginosa* have increased dramatically over the last few years. QS in *P. aeruginosa* is now known to be a complicated interconnected network involving AHL-dependent and AQ-dependent QS systems (Fig. 2). However, there are still many questions that need to be addressed. With respect to AQ biosynthesis, the biochemical roles of PqsABCD, PqsH and PqsL need to be established. The role of PqsE remains an enigma and it is key to know by what mechanism PQS signaling is transduced *via* PqsE. As AQs have been shown to be important for the production of virulence determinants, does AQ signaling provide a target for novel antibacterial agents? The synthesis of inhibitors of AQ-dependent QS could provide ways of tackling *P. aeruginosa* pathogenicity and biofilm formation. Finally, the conservation of AQ signalling amongst different bacterial genera needs to be further investigated. The identification of functional AQ systems in species other than *P. aeruginosa* is exciting, but may only represent the 'tip of the ice-berg'. Detection methods for AQs has improved dramatically and now researchers can look for AQs using sensitive techniques such as LC-MS/MS,[18] or by using simpler but highly effective bioreporter assays.[44,45] In conclusion, there is much scope for future research in AQ signaling in a diverse number of bacterial species.

Acknowledgements

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