Quinolones: from antibiotics to autoinducers

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Abstract

Since quinine was first isolated, animals, plants and microorganisms producing a wide variety of quinolone compounds have been discovered, several of which possess medicinally interesting properties ranging from antiallergenic and anticancer to antimicrobial activities. Over the years, these have served in the development of many synthetic drugs, including the successful fluoroquinolone antibiotics. Pseudomonas aeruginosa and related bacteria produce a number of 2-alkyl-4(1H)-quinolones, some of which exhibit antimicrobial activity. However, quinolones such as the Pseudomonas quinolone signal and 2-heptyl-4-hydroxyquinoline act as quorum-sensing signal molecules, controlling the expression of many virulence genes as a function of cell population density. Here, we review selectively this extensive family of bicyclic compounds, from natural and synthetic antimicrobials to signalling molecules, with a special emphasis on the biology of P. aeruginosa. In particular, we review their nomenclature and biochemistry, their multiple properties as membrane-interacting compounds, inhibitors of the cytochrome bc1 complex and iron chelators, as well as the regulation of their biosynthesis and their integration into the intricate quorum-sensing regulatory networks governing virulence and secondary metabolite gene expression.

Introduction

Quinolones are molecules structurally derived from the heterobicyclic aromatic compound quinoline, the name of which originated from the oily substance obtained after the alkaline distillation of quinine (Gerhardt, 1842). Since the isolation of quinine from Cinchona bark in 1811, many other quinoline derivatives have been isolated from natural sources (Fig. 1). In particular, 2-hydroxyquinoline and 4-hydroxyquinoline, which predominantly exist as 2(1H)-quinolone and 4(1H)-quinolone, respectively, and form the core structure of many alkaloids, were isolated from plant sources. Several different animal and bacterial species also produce compounds of the quinolone class. These differ not only in the varied substitutions in the carbocyclic and heteroaromatic rings but also have other rings fused to the quinoline nucleus. These have been reviewed on a yearly basis by J.P. Michael in Natural Product Reports (Michael, 2008). Some of these naturally occurring quinolones have profound medicinal properties while others have served as lead structures and provided inspiration for the design of synthetic quinolones as useful drugs. For example (Fig. 1), among 2-quinolones, rebamipide is an antiulcer agent and repirinast has antihistamine properties useful in the treatment of allergic asthma (Uchida et al., 1987). While screening compounds for potential cancer chemopreventive properties, casimiroine, isolated from the seeds of Casimiroa edulis, was found to have antimutagenic activity (Ito et al., 1998). Several 4-quinolone alkaloids, mainly isolated from plant and microbial sources, have antimicrobial activity. For example, 2-alkyl-4(1H)-quinolones (AQs) (Fig. 1, compounds 1–4) and 1-methyl-2-[(4Z)-tridecenyl]-4(1H)-quinolone, evocarpine, its structural isomers and unsaturated homologues (Fig. 1, compounds 5–9) isolated from the extracts of Evodia rutaecarpa show antibacterial activity against Helicobacter pylori, which is implicated in the pathogenesis of chronic gastritis, peptic ulcers and gastric cancers (Rho et al., 1999; Hamasaki et al., 2000). The alkaloid 1 shown in Fig. 1 is rather rare as it bears n-decyl, an even number of carbons in the 2-position. Also, no fewer than eight further 4-quinolones (Fig. 1, compounds 10–17) isolated from the fermentation broth of the actinomycete
Pseudonocardia spp. CL38489 are active in inhibiting the growth of H. pylori. The most potent compound is the epoxide (Fig. 1, compound 16), which has a potent bactericidal [Minimal Inhibitory Concentration (MIC) 10 ng mL\(^{-1}\)] and an even more pronounced bacteriostatic effect (MIC 0.1 ng mL\(^{-1}\)) (Dekker et al., 1998). These quinolones are characterized by the presence of a geranyl or oxidized geranyl side chain at C-2 in place of the usual fatty acid-derived alkyl or alkenyl chain normally found in microbial quinolones. The screening of synthetic analogues of quinine for novel antiplasmodial drugs led to the serendipitous discovery of a precursor used in the synthesis of chloroquine, 7-chloroquinoline, which exhibited antimicrobial activities in vitro. Further investigation of this and similar compounds such as the structurally related 1,8-naphthyridones (which are quinolones with a nitrogen atom substituting C-8) resulted in the discovery of nalidixic acid (1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid),...
which was to become the first practical synthetic quinolone antibiotic (Lesher et al., 1962). This rapidly led to the development of several other 4-quinolone-based antibiotics such as oxolinic acid, cinoxacin and flumequine (Fig. 1), used clinically to treat Gram-negative bacterial infections, and later on to second-generation drugs such as norfloxacin and ciprofloxacin (Fig. 1), also effective against some Gram-negative bacteria. All of the quinolone antibiotics are characterized by the presence of a carboxylic acid function at C-3 (Rohlfing et al., 1976; Mardh et al., 1977; Barry et al., 1984; Galante et al., 1985; Aboul-Fadl & Fouad, 1996).

Interest in the antipathogenic properties of common bacteria started with the pioneering work of Louis Pasteur. Notably, in 1877, Pasteur reported that the coinoculation of Bacillus anthracis with other common living bacteria in animals prevented the development of anthrax, when septicaemia could be avoided. This was interpreted, following the idea that ‘life can prevent life’, as being the result of a competition for oxygen (Pasteur & Joubert, 1877). After Emmerich and Pawlowsky were able to prevent the development of anthrax in preinfected rabbits and guinea-pigs by the inoculation of Streptococcus spp., Charles Bouchard reproduced this effect in 1889 with pure cultures of Bacillus pyocyaneus (Pseudomonas aeruginosa) (Bouchard, 1889). Ten years later, in 1899, Rudolf Emmerich and Oscar Löw concluded that P. aeruginosa released an active antibacterial substance into the medium after cell-free preparations from this organism were found to be sufficient to prevent the development of anthrax, and as it was thought to be the result of an enzymatic process, they called it pyocyanase (Emmerich & Löw, 1899). In 1945, this preparation was determined to consist of a mixture of heat-stable compounds that were separated, partially characterized and named the Pyo compounds (Hays et al., 1945). Pyo I–IV, which later were found to be AQs (Fig. 2), presented strong antibacterial activities against Gram-positive organisms, although much less against Gram-negative bacteria, with Pyo II being 10 times more potent compared with the others. However, Pyo II was toxic and ineffective at protecting mice at subtoxic doses against Streptococcus pneumoniae or Mycobacterium tuberculosis infections (Wells et al., 1947).

**Natural antimicrobial quinolones**

In addition to having antimicrobial activity in vitro, the Pyo compounds produced by P. aeruginosa were found to antagonize, under certain conditions, the action of streptomycin and dihydrostreptomycin against Gram-positive bacteria (Lightbown, 1950, 1954). This inhibitory activity was rapidly attributed to Pyo II, which is a mixture of 2-alkyl-4-hydroxyquinoline N-oxides (AQNOs) (Cornforth & James, 1954, 1956; Lightbown & Jackson, 1954). The inhibitory activity of these N-oxides (at concentration ratios with respect to streptomycin of the order of 1:100) was found to correlate with the potent inhibition of electron transport in both heart-muscle and bacterial cells through the cytochrome bc1 segment (ubiquinol:cyanochrome c oxidoreductase) of the respiratory chain (Lightbown & Jackson, 1954, 1956). This is in line with the need for respiration and the transmembrane potential required for bacteria to take up aminoglycoside antibiotics (Hancock, 1962; Damper & Epstein, 1981; Arrow & Taber, 1986; Taber et al., 1987). 2-Heptyl-4-hydroxyquinoline N-oxide (HQNO) acts as a ubiquinone and menaquinone analogue on quinone-reactive cytochrome b enzymes in various organisms (Van Ark & Berden, 1977; Smirnova et al., 1995; Rotherly & Weiner, 1996). The antimicrobial effect of the N-oxides appears to be limited to Gram-positive bacteria and offers an explanation as to how P. aeruginosa becomes the dominant species over Staphylococcus aureus in cystic fibrosis (CF) lung infections (Machan et al., 1992), although additional factors such as pyocyanin, cyanide and N-(3-oxododecanoyl)-L-homoserine lactone have been shown to play a similar role (Qazi et al., 2006; Voggu et al., 2006). Furthermore, long-term exposure of S. aureus to physiological concentrations of HQNO selects for aminoglycoside-resistant, small-colony variants that are typically found in chronic lung infections (Hoffman et al., 2006; Biswas et al., 2009). Interestingly, the formation of S. aureus small-colony variants is the first step towards the development of dual target resistance against fluoroquinolones (Pan et al., 2002). The low in vivo efficacy, combined with the strong toxicity on mitochondrial respiration, prevented the development of AQNOs as therapeutic antibiotics. However, due to its interference with quinone-dependent cytochromes, HQNO became an invaluable reagent for the study of electron transport chains.

A number of quinolones with interesting antimicrobial properties are also produced by various pseudomonads and other microorganisms. For example, under iron limitation, Pseudomonas fluorescens ATCC 17400 produces quinolobactin (8-hydroxy-4-methoxyquinoidal acid, Fig. 3), which acts as a siderophore (Mossialos et al., 2000). Quinolobactin results from the rapid hydrolysis of the precursor molecule 8-hydroxy-4-methoxy-2-quinoilinethiocarboxylic acid (thioquinolobactin), which, as opposed to quinolobactin, has strong antifungal activity against the plant pathogen Pythium debaryanum (Matthijs et al., 2007). Thioquinolobactin is synthesized via a unique pathway from L-tryptophan via xanthurenic acid and sulphurylation by QbsE, a small sulphur carrier protein (Matthijs et al., 2004; Godert et al., 2007). Pseudomonas fluorescens G308, a potential biocontrol strain, produces N-mercapto-4-formylcarboxystyril [Cbs, 4-formyl-1-sulphanyl-2(1H)-quinolone, Fig. 3], a quinolone that contains an unusual N-mercaptoamide functional group and that has strong antifungal properties.
Fig. 2. Structure, IUPAC names and abbreviations of AQ molecules synthesized by Pseudomonas aeruginosa and a synthetic analogue. Both the tautomeric lactam and the phenolic forms of each molecule are shown. Arrows indicate the equilibrium of these molecules as would exist under physiological conditions. Where more than one name exists for a molecule, the IUPAC designation is indicated, although this may not be the nomenclature used most frequently. The compound C1-PQS is a synthetic analogue that is not produced by P. aeruginosa.
against plant pathogens such as *Fusarium* spp., *Cladosporium cucumerinum* and *Colletotrichum lagenarium* (Fakhouri et al., 2001). Although the biosynthetic pathway for Cbs formation has not yet been elucidated, it was suggested that this compound may be derived from AQs produced via a similar biochemical pathway as that in *P. aeruginosa*. The marine bacteria *Pseudomonas bromoutilis* (Wratten et al., 1977) and *Alteromonas* strain SWAT5 (Long et al., 2003) both synthesize 2-pentyl-4-quinolone (PHQ, also called 2-n-pentyl-4-quinolinol) and 2-heptyl-4-hydroxyquinoline (HHQ, also called 2-n-heptyl-4-quinolinol), which were identified as a consequence of their antibacterial activities. PHQ inhibits the growth of cyanobacteria (*Synechococcus*), algae (*Chaetoceros simplex*, *Cylindrotheca fusiformis* and *Thalassiosira weissflogii*) and impacts on particle-associated marine bacterial communities (Long et al., 2003). HHQ and PHQ have antibacterial activity against *Vibrio anguillarum*, *S. aureus*, *Candida albicans* and *Vibrio harveyi* (Wratten et al., 1977). From sponge-associated marine pseudomonads, several other AQs with various substitutions have been identified and appear to have antibacterial, antiplasmodial, antiviral or cytotoxic properties (Debitus et al., 1998; Bultel-Poncé et al., 1999). The obligate aerobic yeast *Yarrowia lipolytica* produces 1-hydroxy-2-dodecyl-4(1H)-quinolone, a potent inhibitor of the alternative NADH:ubiquinone oxidoreductases, which acts as a ubiquinone analogue (Eschemann et al., 2005), an activity reminiscent of the quinoline N-oxides produced by *P. aeruginosa*, which act on the cytochrome *bc*1 complex. In addition, a large number of additional quinoline alkaloids produced by a variety of other microorganisms, plants and animals have been discovered every year (annually reviewed by J.P. Michael), of which the majority still have to be studied with respect to their biological properties. However, as all the natural quinolones described so far lack the 3-carboxy group, which is essential for the binding and blocking of DNA-type IIA topoisomerase complexes, the antibacterial mechanism of action of these compounds remains to be elucidated.

### Synthetic quinolone antibiotics

The practical applications of nalidixic acid (Fig. 1) as an antimicrobial of therapeutic interest became evident soon after it was discovered (Lesher et al., 1962; Ward-McQuaid et al., 1963). Because it is a polar molecule that avidly conjugates to serum proteins, and therefore presents a large volume of distribution, it is inadequate for the systemic treatment of infections. However, both nalidixic acid and its principal 7-hydroxymethyl metabolite that remains active undergo rapid renal excretion and readily accumulate in the urinary tract (Rollo, 1966; Van Bambeke et al., 2005). As nalidixic acid is notably efficient at arresting the growth of common enterobacteria, its principal indication was in the treatment of uncomplicated urinary tract infections (Ward-McQuaid et al., 1963). It is, however, of little use against infections occurring outside of the urinary tract or those that are caused by organisms such as *P. aeruginosa* and Gram-positive pathogens that are intrinsically resistant to the practical therapeutic concentrations of the antibiotic. From the 1980s onwards, there appeared successive generations of antibiotics related to nalidixic acid such as the fluoroquinolones, which, due to substitutions in the molecule, more specifically the addition of a 6-fluoro group, have extended therapeutic spectra and enhanced pharmacokinetic properties. The development of fluoroquinolones (Fig. 1) such as flumequine, norfloxacin and ciprofloxacin (one of the most consumed antibiotic worldwide; Ruiz, 2003) extended the spectrum of activity of quinolone antibiotics against infections caused by a variety of otherwise resistant organisms such as *P. aeruginosa* and both aerobic and anaerobic Gram-positive pathogens, and enabled the treatment or the prevention of more severe conditions such as renal, respiratory, abdominal and sexually transmitted bacterial infections (for an extensive review on quinolone antibiotics, see Van Bambeke et al., 2005).

Quinolone antibiotics act by inhibiting the two type IIA bacterial topoisomerases: DNA gyrase and topoisomerase IV (bacterial type IIA topoisomerases have been reviewed recently by Sissi & Palumbo, 2010). DNA gyrase is a heterotetramer formed by two subunits encoded by *gyrA* (*nalA*) and *gyrB* (*nalC*). GyrA together with GyrB acts by creating DNA gates or double-stranded gaps in the DNA through which the strands are passed, introducing negative supercoils into DNA and relaxing the positive supercoiling resulting from replication as the strands unwind.
(Champoux, 2001; Wang, 2002; Corbett & Berger, 2004; Leo et al., 2003; Drlica et al., 2009). Nalidixic acid and oxolinic acid, a more potent, but structurally similar quinolone antibiotic (Staudenbauer, 1976), were initially found to inhibit in vitro the supercoiling activity of purified DNA gyrase (Gellert et al., 1977; Sugino et al., 1977). Whereas only gyrase is able to introduce negative supercoiling, the function of DNA topoisomerase IV, a heterotetramer formed by two ParC-ParE subunits similar to the GyrA-GyrB subunits of DNA gyrase, is essential for the relaxation of supercoiled DNA and the resolution of catenated DNA molecules after replication (Kato et al., 1990, 1992; Adams et al., 1992; Peng & Marians, 1993; Hoshino et al., 1994; Chen et al., 1996). The precise mode of action of the quinolone antibiotics on type IIA topoisomerases has long been debated. However, crystallographic studies strongly suggest that these molecules essentially act by blocking the DNA–topoisomerase complexes when the nucleic acid is cleaved (Chen et al., 1996; Laponogov et al., 2009). Thus, in addition to sharing structural and functional similarities, both DNA gyrase and topoisomerase IV can be inhibited by quinolone antibiotics, leading to bacterial cell death due to chromosome fragmentation. Whereas the target of quinolone antibiotics in Escherichia coli and other Gram-negative bacteria is mainly DNA gyrase, in Gram-positive species such as S. aureus or S. pneumoniae, their principal mode of action lies mainly in the inhibition of topoisomerase IV, with exceptions depending on the particular fluoroquinolone compound and bacterial species (Higgins et al., 2003; Eliopoulos, 2004; Laponogov et al., 2009).

Resistance to quinolone antibiotics (reviewed by Jacoby, 2005 and by Martinez et al., 2009a) can be achieved by three nonexclusive mechanisms: (1) by acquisition of point mutations in the genes encoding either of the two type IIA topoisomerases targeted, DNA gyrase and DNA topoisomerase IV, (2) by reducing the effective concentrations of the drugs in the cytoplasm, either passively by alterations in the membrane permeability or actively by overexpressing efflux systems, and (3) by acquisition of mobile quinolone resistance determinants (Ruiz, 2003; Jacoby, 2005). While only target modification confers high-level resistance to quinolone antibiotics, the low-level resistance (less than a 10-fold increase in the minimum inhibitory concentration) conferred by the other mechanisms augments the probability of developing such mutations. Mutants that exhibit more than a 100-fold decrease in quinolone sensitivity have been found to carry single point mutations in the chromosomally encoded DNA gyrase subunit gene gyrA, with additional enhanced resistance when certain point mutations are simultaneously present in the distantly located gyrB gene (Yamagishi et al., 1986; Yoshida et al., 1988; Cullen et al., 1989; Yoshida et al., 1990, 1991; Oram & Fisher, 1991; Cambau et al., 1993; Ruiz, 2003). Molecular details of these mutations and the binding of quinolones to DNA–topoisomerase complexes have been described elsewhere extensively (Ng et al., 1996; Cabral et al., 1997; Hiasa & Shea, 2000; Friedman et al., 2001; Jacoby, 2005).

In Gram-negative bacteria, a reduction in antibiotic permeability can be achieved by altering the cell envelope. For example, E. coli or P. aeruginosa clinical isolates that produce altered lipopolysaccharides differ in the accumulation of quinolones compared with wild-type strains, a nonspecific mechanism thought to involve changes in surface hydrophobicity and consequently affecting passive drug diffusion (Cohen et al., 1989; Leying et al., 1992; Everett et al., 1996; Chenia et al., 2006). However, a reduction in permeability to quinolones is most often achieved by disrupting or downregulating a number of outer-membrane proteins that form channels through which quinolones enter the bacterial cell or by the activation of tripartite multidrug efflux systems belonging to the AcrAB-TolC resistance-nodulation-division (RND) family that can prevent quinolone antibiotics reaching effective concentrations in the cytoplasm (reviewed by Martinez et al., 2009b). For instance, P. aeruginosa encodes at least 12 RND systems (Stover et al., 2000; Schweizer, 2003), eight of which (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM, MexHI-OpmD, MexVW-OprM and MexPQ-OpmF) have been reported to export fluoroquinolones and other antibiotics (Chuanchuen et al., 2002; Li et al., 2003; Sekiya et al., 2003; Van Bambeke et al., 2003; Mima et al., 2005), although antimicrobial resistance does not appear to be their primary biological function (Aeschlimann, 2003; Van Bambeke et al., 2003; Poole, 2004, 2008).

Resistance to quinolone antibiotics can also result from the acquisition of plasmid-borne determinants. The MFS-type efflux pump QepA and the Aac(6′)-Ib-cr enzyme that confers decreased susceptibility to piperazinyl fluoroquinolones such as ciprofloxacin and norfloxacin by acetylation are examples of recently discovered resistance genes carried by plasmids (Robicsek et al., 2006; Périchon et al., 2007; Yamane et al., 2007). More frequent, however, are the plasmids carrying Qnr quinolone resistance loci, of which at least five families have been identified, mostly in Enterobacteriaceae (Jacoby et al., 2008; Cavaco et al., 2009; Wang et al., 2009). These genes encode proteins of the pentapeptide repeat family that interact with DNA gyrase and topoisomerase IV, preventing quinolone inhibition by mimicking DNA, which probably reduces the availability of holoenzyme–DNA targets for quinolone inhibition (Tran & Jacoby, 2002; Hegde et al., 2005; Tran et al., 2005a,b). As with the other quinolone resistance determinants, this function may be considered biologically fortuitous because natural quinolones inhibiting type IIA topoisomerases have not been discovered.
Quinolones produced by *P. aeruginosa*

Besides Pyo II, which is a 2:1 mixture of HQNO and 2-nonyl-4-hydroxyquinoline N-oxides (NQNO) (Lightbown, 1950, 1954), with small quantities of 2-undecyl-4-hydroxyquinoline N-oxide (UQNO) (Cornforth & James, 1956), *P. aeruginosa* also releases a large number of related molecules. Using ozonolysis and UV absorption spectra in comparison with synthetic standards, Wells et al. (1952) identified Pyo Ib as 2-heptyl-4(1H)-quinolone (HHQ), Pyo Ic as 2-nonyl-4(1H)-quinolone (NHQ) and Pyo III as a monounsaturated alkyl side chain variant of NHQ (Wells, 1952; Wells et al., 1952).

The AQ biosynthetic enzymes of *P. aeruginosa* enable this organism to generate a diverse range of related AQ molecules (Fig. 2 and Box 1). An early study using GC and electron capture MS identified over 20 different AQS (Taylor et al., 1995), with HHQ being the most prevalent, followed by NHQ. Variations of these compounds containing saturated and monounsaturated alkyl side chains varying from one to 13 carbons in length, and the two major N-oxides, HQNO and NQNO, were also found. Two subsequent studies used electrospray ionization and LCMS to obtain the mass spectra of over 50 different AQS. These mainly consisted of 2-heptyl-3-hydroxy-4(1H)-quinolone [termed the *Pseudomonas* quinolone signal (PQS)], HHQ, HQNO and NHQ, with several other saturated and monounsaturated alkyl side chains of various lengths (Lépine et al., 2003, 2004). Additional AQS that have been found in significant amounts are 2-nonyl-3-hydroxy-4(1H)-quinolone (C9-PQS), 2-undecyl-4-hydroxyquinoline (UHQ), NQNO and UQNO (Taylor et al., 1995; Déziel et al., 2004; Lépine et al., 2004). Several variations of these compounds are produced (Fig. 2 and Box 1), but many at seemingly biologically insignificant levels, perhaps as a consequence of a lack of specificity of the AQ biosynthetic enzymes for β-keto fatty acids of different chain lengths rather than for any particular biological function. In addition, a metabolite identified as 2,4-dihydroxyquinoline (DHQ) was found in cultures of both *Pseudomonas* and *Burkholderia thailandensis* (Lépine et al., 2007; Zhang et al., 2008). DHQ, although structurally related, is technically not an AQ as it lacks a 2-alkyl chain. It is neither a degradation product nor a precursor of AQS and the precise function of this molecule remains unknown.

**Properties of AQS**

Generally, AQS have a low aqueous solubility. For example, the solubility of PQS is around 1 mg L\(^{-1}\) (~5 μM) in water (Lépine et al., 2003). Because of this hydrophobic nature, a

Box 1. Nomenclature and abbreviations of AQS used in this review

<table>
<thead>
<tr>
<th>Suggested nomenclature</th>
<th>Synonyms</th>
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<tr>
<td>2-Alkyl-4(1H)-quinolone (AQ)</td>
<td>2-Alkyl-4-hydroxyquinolone (AHQ)</td>
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<tr>
<td>2-Alkyl-4-hydroxyquinolone</td>
<td>4-hydroxy-2-alkylquinolone (HAQ)</td>
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<tr>
<td>N-oxide (AQNO)</td>
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</tr>
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<td>2-Heptyl-3-hydroxy-4(1H)-quinolone</td>
<td>2-Alkyl-1-hydroxy-4(1H)-quinolone</td>
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<tr>
<td><em>Pseudomonas</em> quinolone signal</td>
<td>2-Heptyl-3,4-dihydroxyquinolone</td>
</tr>
<tr>
<td>PQS</td>
<td>2-Heptyl-3,4-quinolinediol</td>
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</table>

The structures, IUPAC-based nomenclature and abbreviations of all the major AQS produced by *Pseudomonas aeruginosa* are summarized in Fig. 2. Some non-IUPAC names that have been used to describe some of these same molecules in the scientific literature have been included for clarity. AQS, 2-alkyl-4(1H)-quinolones (lactam form) are tautomeric with 2-alkyl-4-hydroxyquinolines (phenolic form), of which the predominance of one form over the other is determined by the pH (Katritzky & Lagowski, 1963; Katritzky et al., 1991; Larsen, 2005). For example, it has been demonstrated using pH\(_5\) values for 2-methyl-3-hydroxy-4(1H)-quinolone (C1-PQS) that over physiological pH ranges, the neutral 4-quinolone form is the predominant tautomer (Diggle et al., 2007). These tautomeric forms are shown in Fig. 2, with their relative ratios indicated by the arrows. Ideally, for consistency and structural accuracy, nomenclature and abbreviations based on only one tautomeric form should have been uniformly adopted. However, this causes some difficulties because in the available scientific literature individual research groups have subjectively referred to these molecules in either one form or the other. For example, even the names used for the two main AQ molecules involved in signalling, PQS and HHQ, are inconsistent with each other with regard to tautomerism: *Pseudomonas quinolone* signal and 2-heptyl-4-hydroxyquinolone. The nomenclature and abbreviations used in this review therefore amount to a compromise between what is technically correct, taking into account IUPAC designations and structural predominance due to physiological pH, and also what has been the prevalent terminology used in the scientific literature for each molecule. Hence, the abbreviation PQS to designate 2-heptyl-3-hydroxy-4(1H)-quinolone has been maintained and the alkyl side chain variants of this molecule abbreviated by the number of carbon atoms in the side chain, for example C1-PQS, C9-PQS. The designation HHQ for 2-heptyl-4-hydroxyquinolone has also been maintained, and the other alkyl side-chain derivatives have been abbreviated accordingly (e.g. PHQ for 2-pentyl-4-hydroxyquinolone, etc.). It should be noted that the N-oxide series of compounds (AQNOs, e.g. HQNO, 2-heptyl-4-hydroxyquinolone N-oxide; NQNO, 2-nonyl-4-hydroxyquinolone N-oxide) can adopt the 2-alkyl-1-hydroxy-4(1H)-quinolone form (Fig. 2) but not at physiological pH. DHQ or 2,4-dihydroxyquinolone can exist in both, 4-hydroxy-2(1H)-quinolone (predominant at physiological pH) and 2-hydroxy-4(1H)-quinolone tautomeric forms (Fig. 2), but to avoid confusion and to conform to the literature citations, DHQ is used to denote this molecule in this review. To further help the reader, below is a list of the proposed nomenclature of the AQS that are mentioned along with their abbreviations. Included in this table are the associated synonyms that have been used to describe these same molecules elsewhere in the scientific literature.
Box 1. Continued.

<table>
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<th>Suggested nomenclature</th>
<th>Synonyms</th>
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<tr>
<td>3-hydroxy-2-nonyl-4(1H)</td>
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<td>-quinolone (C9-PQS)</td>
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<td>2-pentyl-4-hydroxyquinoline</td>
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<td>(UHQ)</td>
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high proportion of the AQs are associated with the bacterial outer membrane and with membrane vesicles (MV)s (Mashburn-Warren et al., 2008). Of the total amount of PQS produced by P. aeruginosa PA14, around 80% appears to be contained within vesicles, in contrast with < 1% of either of the P. aeruginosa N-acetyl-homoserine lactone (AHL)-based signal molecules N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) (Mashburn & Whiteley, 2005). The PQS contained within these MVs is seemingly both bioactive and bioavailable because the addition of MVs containing PQS restored the production of pyocyanin in a PQS-negative mutant (PQS being indispensable for the production of pyocyanin in P. aeruginosa). The MVs themselves do not seem to have any direct effect on the production of pyocyanin and PQS does not need to be packaged into MVs to exert its effects. MV formation in P. aeruginosa PA14 would not seem to be an active process as it occurs independent of growth or of protein synthesis (Mashburn & Whiteley, 2005). Instead, PQS appears to initiate the formation of MVs, into which it is then packaged due to its lipophilic nature (Mashburn & Whiteley, 2005). A mechanism for MV formation has been proposed via the interaction of PQS with the 4’-phosphate and acyl chain of bacterial lipopolysaccharide (Mashburn-Warren et al., 2008). Because HHQ is much less efficient in inducing vesicle formation, this activity seems to be dependent on the 3-hydroxy group of PQS and its analogues (Mashburn-Warren et al., 2008, 2009). A pqsH mutant, deficient in the conversion of HHQ to PQS, is defective in vesicle formation. Of PQS and its analogues, MV formation appears to be optimal when a C7 2-alkyl side chain moiety is present, although C5 and C3 alkyl side chain variants also exhibit some activity and MVs can still be induced to some extent by PQS analogues lacking a 2-alkyl side chain, indicating that this group is dispensable. Additionally, compounds that can inhibit PQS production such as indole and its derivatives reduce MV formation, presumably as there is less PQS available to induce vesicle formation (Tashiro et al., 2010). It has been suggested that packaging into MVs could protect PQS from degradation by surrounding cells or competing microbial communities. Arthrobacter nitroguajalicolicus RUS1 produces the cytoplasmic enzyme Hod [3-hydroxy-2-methyl-4(1H)-quinolone 2,4-dioxygenase], which catalyses the 2,4-dioxygenolytic ring cleavage of PQS with the concomitant formation of carbon monoxide and N-octanoyl-anthranilic acid (Pustelnik et al., 2009). As purified Hod is capable of inhibiting AQ signalling when added to cultures of P. aeruginosa, at present, the extent of protection conferred by MVs to AQs against enzymatic degradation is unclear.

Rhamnolipids are produced by P. aeruginosa and act as biosurfactants, facilitating swarming motility (Caiazza et al., 2005). However, rhamnolipids also enhance the aqueous solubility and activity of AQs in vitro. The addition of increasing amounts of rhamnolipids enhanced the ability of PQS at a range of concentrations to induce the expression of a lasB-lacZ translational reporter, suggesting that in P. aeruginosa the induction of elastase production by PQS is enhanced in the presence of rhamnolipids (Calfée et al., 2005). Whether rhamnolipids are indeed effectively utilized to solubilize PQS in vivo is, however, not known at present, and with respect to the above reporter system, an excess of rhamnolipids even seems to be detrimental to its expression. A reason for this may be that above a certain threshold concentration, PQS is sequestered into rhamnolipid micelles and therefore becomes less available to the cells (Calfée et al., 2005).

AQNOs such as HQNO are potent inhibitors of the cytochrome bc1 complex and an interesting, but as yet undescribed facet is the mechanism by which P. aeruginosa avoids self-poisoning as a consequence of the endogenous production of these molecules. Gram-negative bacteria, as opposed to Gram-positive species such as Bacillus subtilis or S. aureus, are normally resistant to these compounds, and possible explanations for this have been (1) a reduced cell wall permeability, (2) enzymatic inactivation or (3) an active efflux system to transport the molecule out of the cells (Machan...
et al., 1992). However, none of these mechanisms can account for the resistance of P. aeruginosa towards endogenously produced HQNO. Aerobic respiration in P. aeruginosa is achieved by a branched electron transport chain ending in five different terminal oxidases, three of which (cytochrome oxidases cbb3, 1, cbb3, 2 and aa3) receive electrons from ubiquinone via the cytochrome bc1 complex and cytochromes c, whereas the remaining two are the cytochrome bo3 and the cyanide-insensitive cytochrome bd quinol oxidases, which bypass the cytochromes bc1-c electron transfer pathway and get their electrons directly from ubiquinone (Williams et al., 2007a). Thus, if the cytochrome bc1 complexes of P. aeruginosa were sensitive to HQNO, this compound alone would have the potential to inhibit three out of five electron transport chains, and in combination with the production of cyanide, 80% of aerobic respiration would be inhibited. Pseudomonas aeruginosa is also able to perform anaerobic respiration using nitrogen oxides as terminal electron acceptors. Nitrite, nitric oxide (NOR) and nitrous oxide terminal reductases receive electrons from the cytochrome bc1 complex, while nitrate reductase (NAR) obtains electrons directly from ubiquinone and also in part via a dedicated membrane-bound formate dehydrogenase (Williams et al., 2007a). In this case, HQNO could potentially block all the nitrogen oxide anaerobic respiration, except for the NAR respiratory chain involving formate oxidation. A recent study reported that abolishing AQ production in P. aeruginosa enhanced anaerobic growth on nitrate, and that addition of PQS appeared to repress the growth of the wild type and inhibited denitrifying enzymes (Toyofuku et al., 2008). Although this effect was attributed to the iron-chelating properties of PQS, the involvement of increased HQNO production cannot be excluded. Whether P. aeruginosa prevents self-poisoning with HQNO under aerobic conditions by favouring the cytochrome bo3 and/or the cyanide-insensitive cytochrome bd oxidase pathways or whether there are mechanisms to prevent competitive inhibition of ubiquinone-dependent enzymes under aerobic and anaerobic growth on nitrate remains to be determined.

**Biosynthesis of AQs in P. aeruginosa**

AQ biosynthesis requires multiple genes, which were initially identified by screening a P. aeruginosa transposon mutant library for clones displaying reduced quinolone production and were termed pqsABCDE, pqsR (mvrR), pqsH and pqsL (Cao et al., 2001; D’Argenio et al., 2002; Gallagher et al., 2002; Lépine et al., 2002). The pqsABCDE (PA0996-PA1000) genes are arranged in an operon, and adjacent to these are the anthranilate synthase genes phnAB (PA1001-PA1002) and pqsR (mvrR, PA1003). Two other genes are also involved in AQ biosynthesis, pqsH (PA2587) and pqsL (PA4190), but both of these are located separately elsewhere on the chromosome.

The pqsR gene encodes a LysR-type transcriptional regulator that has a helix-turn-helix motif at the N-terminus with the first 280 amino acids sharing high similarity (62–71%) with other LysR-type regulators (Cao et al., 2001; Maddocks & Oyston, 2008). PqsR is the transcriptional regulator of both the pqsABCDE and the phnAB operons and is of crucial importance for AQ production. A mutation in the gene coding for this regulator in P. aeruginosa strain PA14 resulted in the abolition of phnAB and pqsABCDE transcription along with PQS and AQ biosynthesis and had corresponding effects on other virulence determinants including pyocyanin, elastase, exoprotein and 3-oxo-C12-HSL production and consequently the reduced ability to cause disease in plants and animals (Cao et al., 2001; Déziel et al., 2004).

The pqsABCD genes are involved in the biosynthesis of all AQs (Déziel et al., 2004). The first step in this biosynthesis involves the activation of anthranilate by PqsA, an anthranilate coenzyme A ligase (Coleman et al., 2008). PqsB and PqsC, which are similar to β-ketoacyl-ACP (acyl carrier protein) synthases involved in fatty acid metabolism, are predicted to elongate acyl side chains of AQ precursors. However, little is currently known about the enzymatic functions of these proteins. PqsD shares some sequence similarity with the Cys-His-Asn active site of the E. coli initiation condensing enzyme FabH (Luckner & Ritter, 1965; Ritter & Luckner, 1971; Bredenbruch et al., 2005; Zhang et al., 2008). The crystal structure of PqsD with and without a potential covalently bound anthranilate-AQ intermediate product has been resolved recently (Bera et al., 2009). Nonpolar mutations in either the pqsA, pqsB or pqsD genes completely abolish the production of AQs (Diggle et al., 2003; Zhang et al., 2008).

In addition to these four AQ biosynthesis genes, the pqsABCDE operon also encodes PqsE, which has sequence similarities to proteins of the metallo-β-hydrolase superfamily. This extensive family of hydrolytic enzymes mediates a wide range of functions, such as β-lactamases, glyoxalases, AHL-lactonases and arylsulphatases. These enzymes are usually characterized by a conserved metal ion-binding HXXHDXH amino-acid motif, which is also found in PqsE and that forms an active site able to bind two iron atoms (Yu et al., 2009). However, although the PqsE crystal structure is available, little knowledge has been gained about its exact function, its natural substrate remaining unknown. The deletion of pqsE reduces the production of several virulence factors including pyocyanin, lecin and hydrogen cyanide (HCN), while overexpression of pqsE has the opposite effect. A transcriptomic analysis has recently revealed that the abundances in the mRNAs of ≈400 genes depend on the level of expression of pqsE, and that virulence in plant and animal infection models in the absence of AQ depends on this gene (Rampioni et al., 2010). The activity of
PqsE has also been reported to be dependent on RhlR, which acts downstream, but in synergy with PqsE (Hazan et al., 2010). Interestingly, PqsE is not involved in AQ biosynthesis, and appears to be a crucial element mediating the cellular response to PQS to achieve full virulence (Gallagher et al., 2002; Diggle et al., 2003; Déziel et al., 2005; Farrow et al., 2008).

The *pqsH* gene encodes a predicted FAD-dependent monooxygenase that hydroxylates the 3' carbon atom of HHQ in the final step of PQS biosynthesis. As such, *pqsH* mutants do not produce 3-hydroxylated quinolones, but continue to produce other AQS (Déziel et al., 2004). Because *pqsH* is regulated by LasR, but not by PqsR, it is therefore conceivable that under certain circumstances, a differential regulation of these two elements may lead to the overproduction of HHQ with respect to PQS, due to a lack of PqsH (Déziel et al., 2004).

The *pqsL* gene encodes a second, distinct monooxygenase that is required for the synthesis of HQNO and related N-oxides via oxidation of the quinolone ring nitrogen atom. The detailed mechanism of AQNO biosynthesis is still unknown, but interestingly, HHQ does not appear to be a precursor of HQNO, as the addition of deuterated HHQ to a culture of *P. aeruginosa* resulted in the biosynthesis of deuterated PQS, but not of deuterated HQNO (Déziel et al., 2004). Additionally, a *pqsL* mutant overproduces PQS compared with its isogenic wild-type parent, suggesting that PqsL interacts with and diverts a fraction of the HHQ precursor products towards AQNO biosynthesis and away from HHQ and PQS biosynthesis (D’Argenio et al., 2002).

A simplified scheme for the biosynthesis of AQS is detailed in Fig. 4. Before the role of these molecules in signalling was discovered, an AQ biosynthesis pathway had already been proposed. This was based on radiolabelled precursor feeding experiments, which indicated condensation of anthranilic acid with β-keto fatty acids, releasing CO₂ and H₂O (Cornforth & James, 1956; Luckner & Ritter, 1965; Ritter & Luckner, 1971). This was confirmed more recently by MS and nuclear magnetic resonance (NMR) analysis of the AQS produced after feeding 13C and 15N isotope-labelled precursors to *P. aeruginosa* (Bredenbruch et al., 2005). This study also ruled out a second possible pathway of AQ biosynthesis that involved the formation of a kynurenic acid precursor resulting from a reaction between

![Fig. 4. Proposed biosynthetic pathway of PQS, HHQ, HQNO and DHQ in *Pseudomonas aeruginosa*. AQS are derived from a condensation reaction between anthranilate and β-keto fatty acids. Anthranilate is derived from either the PhnAB/TrpEG or the KynABU metabolic pathways using either chorismate or tryptophan as precursors, respectively. Anthranilate is first activated with coenzyme A (CoA) by PqsA. Anthranilate-CoA and an activated β-ketodecanoate are condensed, possibly via the PqsBCD enzymes to HHQ, releasing CO₂ and H₂O. The monooxygenase PqsH converts HHQ to PQS. HQNO is derived from the same starting products as HHQ, but utilizes the additional monooxygenase PqsL. HHQ is not a precursor for HQNO. DHQ, which technically is not an AQ, is produced by PqsD independent of PqsB and PqsC.](https://academic.oup.com/femsre/article-abstract/35/2/247/660032)
orotic acid and anthranilate. The heterologous expression of AQ biosynthesis genes in *E. coli* revealed that the production of DHQ (Fig. 2) only requires PqsA and PqsD (Zhang et al., 2008). Activated anthraniloyl-CoA, generated by PqsA, is transferred to the cysteine residue in the active site of PqsD (unactivated anthranilate does not transfer). Here, it reacts with either malonyl-CoA or malonyl-ACP to form 3-(2-amino-phenyl)-3-oxopropanoyl-CoA, a short-lived intermediate that undergoes an internal rearrangement to form DHQ. Some variation of this biosynthesis, utilizing longer chain β-keto fatty acids in place of malonyl-CoA or malonyl-ACP, is possibly the mechanism by which AQ molecules such as HHQ are produced. However, because the above process only utilizes PqsA and PqsD, AQ biosynthesis is likely to be more complex as the functions of PqsB and PqsC are still unclear (Zhang et al., 2008; Bera et al., 2009).

The substrates required for AQ biosynthesis have also been investigated. Two pairs of genes responsible for anthranilate biosynthesis had been described previously: *phnAB* (Essar et al., 1990a), located adjacent to the *pqs* operon (Gallagher et al., 2002), and *trpEG*, which encode enzymes involved in tryptophan biosynthesis (Essar et al., 1990b). The *phnAB* genes code for proteins resembling the *E. coli* anthranilate synthase subunits TrpE and TrpG (Essar et al., 1990a) and are cotranscribed by PqsR (Cao et al., 2001). It was initially thought that these would provide anthranilate as a precursor for phenazine biosynthesis as inactivation of these genes reduced pyocyanin production. However, it was subsequently shown that PhnA and PhnB do not appear to be involved in this function (Mavrodi et al., 2001) and therefore the reduction in pyocyanin production in a *phnAB* mutant is instead most likely to be the consequence of the reduced availability of AQS. In addition to PhnA and PhnB, TrpE and TrpG also direct the synthesis of anthranilate from chorismate, which can then be utilized either for tryptophan or for AQ biosynthesis. A third source of anthranilate comes from the homologues of the tryptophan 2,3-dioxogenase KynA, the kynurenine formamidase KynB and the kynureninase KynU to produce anthranilate from tryptophan (Kurnasov et al., 2003; Farrow & Pesci, 2007). Detailed analyses indicate that in rich growth media containing the aromatic amino acid tryptophan, the kynurenine pathway is the main source of anthranilate for AQ production, whereas the *phnAB* genes supply anthranilate in minimal media in the absence of exogenous tryptophan (Farrow & Pesci, 2007). Interestingly, increased PQS production by *P. aeruginosa* strains isolated from infected CF lungs has been correlated with the presence of aromatic amino acids in the growth medium (Palmer et al., 2005). In this case, the transcription of *pqsA* was found to be induced by tryptophan, phenylalanine and tyrosine, while the nonaromatic amino acid serine had little effect. The kynurenine pathway may therefore be the principal source of anthranilate in a lung infection context.

The requirement of anthranilate for PQS production has been demonstrated. When *P. aeruginosa* PAO1 parent and isogenic las QS mutants unable to produce PQS were grown in the presence of anthranilate labelled with 14C in the heteroaromatic ring, most of the radioactivity was found in the AQ extracts for those strains able to generate PQS, whereas very little was found in the supernatant extracts of the QS mutants (Calfee et al., 2001). This suggested that the strains not producing PQS would incorporate anthranilate, but not convert it into AQS. Additionally, when *P. aeruginosa* was grown with increasing amounts of methyl-anthranilate, PQS biosynthesis levels were reduced as this compound acted as a competitor of anthranilate (Calfee et al., 2001). The production of elastase, which is dependent on PQS signalling, was also inhibited by methyl-anthranilate in a concentration-dependent manner. At 1.5 mM, methyl-anthranilate practically abolished elastase production, the suggested consequence of a much reduced level of PQS production.

Feeding experiments with isotope-labelled AQ precursors such as 15N-anthranilate coupled with GC–MS analysis resulted in the production of AQs having incorporated around 66% of 15N, further demonstrating that anthranilate serves as a common precursor for AQS and that the heteroaromatic nitrogen in the quinolone ring originates from this molecule (Bredenbruch et al., 2005). Similarly, feeding labelled 13C-acetate to *P. aeruginosa* PAO1 demonstrated that the heteroaromatic ring of the quinolone moiety was formed from acetate. The resulting GC–MS fragmentation pattern, together with confirmation by NMR spectroscopy, indicated that the mechanism of this reaction was via a direct head-to-head reaction involving anthranilate and β-keto fatty acids derived from acetate (Bredenbruch et al., 2005). β-Keto fatty acids are therefore essential precursors in the biosynthesis of AQS. Some studies had suggested that there is a link between rhamnolipid biosynthesis and AQ production, which was interesting because rhamnolipids are composed of a rhamnose moiety and fatty acids of the same chain lengths as those involved in AQ biosynthesis. Rhamnolipids have also been shown to increase PQS solubility and may mediate this function in vivo (Calfee et al., 2005). It was initially thought that *rhlG* coded a potential β-ketoacyl-ACP reductase that could participate in the provision of fatty acids utilized as a substrate for AQ biosynthesis (Bredenbruch et al., 2005) as RhlG was assumed to direct the incorporation of these fatty acids into rhamnolipids (Campos-García et al., 1998; Déziel et al., 2003; Sobrón-Chávez et al., 2005). However, recent studies have contradicted this, as an *rhlG* mutant was unaltered in rhamnolipid production compared with the corresponding wild type (Zhu & Rock, 2008). Furthermore, the crystal
structure of RhlG revealed that its function was inconsistent with the proposed fatty acid biosynthetic pathway (Miller et al., 2006). Therefore, it appears that RhlG is not involved in rhamnolipid or AQ biosynthesis.

In *P. aeruginosa*, PQS is likely to be the end product of the AQ synthetic pathway or is not substantially converted into other molecules, as when labelled PQS was added to wild-type cultures, no additional compounds could be identified (Déziel et al., 2004).

**Quorum sensing (QS) and AQ production in *P. aeruginosa***

When favourable nutritional conditions are encountered, bacteria will proliferate to form established multicellular communities that have the potential to adapt to and modify their environment. This allows further exploitation of nutrient resources that would otherwise be restricted for individual cells. The mechanism by which a bacterium adapts from the lifestyle of an individual cell to a community capable of modifying their environment has been termed QS and it is defined as a mechanism by which bacteria regulate specific target genes in response to a critical concentration of endogenously produced signal molecules dedicated to the probing of the cell population density (Venturi, 2006; Williams et al., 2007b). This process is mediated by the production and sensing of autoinducers, small signalling molecules, whose concentration in the extracellular medium reflects cell population density. *Pseudomonas aeruginosa* produces two AHLs as QS signal molecules, each acting as the autoinducer of a specific sensing and responding system: 3-oxo-C12-HSL acts on the las system and C4-HSL acts on the rhl system. The core of each system is composed of a synthase producing an AHL for the activation of a specific transcriptional regulator: LasI produces 3-oxo-C12-HSL for the activation of LasR (Gambello & Iglewski, 1991; Passador et al., 1993; Pearson et al., 1994) and RhlI produces C4-HSL for the activation of RhlR (Latifi et al., 1995; Pearson et al., 1995; Winson et al., 1995). Initially, each synthase gene is expressed at basal levels and the AHLs produced diffuse into the surrounding medium. Autoinduction is achieved when the accumulation of an AHL reaches a threshold concentration and the activated transcriptional regulators LasR and RhlR further enhance the expression of the synthase genes lasI and rhlI, respectively, generating positive feedback loops (Seed et al., 1995). When the transcriptional regulators are activated they will induce the transcription of overlapping subsets of genes. For example, LasR will induce the production of virulence factors such as elastase (Passador et al., 1993) and pyoverdin (Stintzi et al., 1998), while RhlR will increase the production of rhamnolipid biosurfactants (Ochsner & Reiser, 1995), cytotoxic lectins, pyocyanin and elastase, among other virulence factors (Pearson et al., 1997). In addition to some overlap between the genes targeted by both AHL QS systems due to the similarities of the palindromic las/rhl boxes recognized by LasR and RhlR (Schuster et al., 2004; Schuster & Greenberg, 2006, 2007), activated LasR will also induce the rhl system (Latifi et al., 1996), creating a hierarchical regulatory network, which in turn is further modulated by additional regulatory elements (reviewed in von Bodman et al., 2008 and in Williams & Câmara, 2009).

Besides the AHL-based QS systems, *P. aeruginosa* utilizes an autoinducer regulatory system based on the AQS. This system relies on the PQS and its precursor molecule HHQ to control global gene expression (Pesci et al., 1999; Déziel et al., 2004). The transcriptional regulator PqsR controls the expression of the pqsABCDE and phnAB biosynthetic operons and therefore pqsR is essential for the production of AQS (Gallagher et al., 2002; Déziel et al., 2004; McGrath et al., 2004).

The pqsR gene is convergently transcribed with respect to the pqsABCDE-phnAB operons and two transcriptional start sites have been mapped 190 and 278 bp upstream of its start codon (Wade et al., 2005). The distant promoter appears to have a typical σ^70-binding site signature, indicative of basal transcription, and a putative las/rhl box operator sequence is found centred 239–258 bp upstream of this transcriptional start site (517–536 bp upstream of the start codon). *In vitro*, PqsR binds at two different locations upstream of pqsA, and the strength and position of the binding depend on the presence of PQS (Wade et al., 2005). The pqsA transcriptional starting point has been mapped 71 bp upstream of the start codon (McGrath et al., 2004). Alterations of a LysR-type box located at −45 in the pqsA promoter can result in the loss of PqsR-binding capacity and in the reduction of transcription initiation, suggesting that this element plays a central role in the regulation of the pqsABCDE operon by PqsR and PQS (Xiao et al., 2006b). Overexpression of pqsR strongly repressed the transcription of antA, which encodes an anthranilate 1,2-dioxygenase. This is thought to ensure an adequate supply of anthranilate for the biosynthesis of AQS by reducing its metabolic degradation (Oglesby et al., 2008).

When the pqsABCDE operon and pqsR were cloned in *E. coli* and expressed from their native promoters, HHQ and NHQ were produced, but not PQS because *E. coli* lacks a pqsH homologue. Similarly, compared with the wild type, the activity of the pqsA promoter and AQ production levels (except for PQS) remained comparable when pqsH was disrupted. This indicates that in addition to PQS, other AQS can also act as autoinducers (Xiao et al., 2006a). It has been suggested that HHQ induces a conformational change in PqsR, as binding of PqsR to the pqsA promoter in *vitro* is enhanced by HHQ, although not as much as with PQS. In an AQ-negative double pqsA pqsH mutant derived from
strains PAO1 or PA14, PQS was found to be 100 times more potent at inducing the pqsA promoter than HHQ (Xiao et al., 2006a; Diggle et al., 2007). In strain PA14, the deletion of pqsH reduced the overall expression of the pqsR regulome by less than twofold, and the addition of exogenous PQS to this mutant did not revert the expression levels of this regulome substantially above wild-type levels, further implying a role for HHQ in inducing many of the genes. An exception to this was phaA1, as PQS appears to be essential for the transcription of this gene and for the production of pyocyanin (Xiao et al., 2006a). Altogether, these studies indicate that HHQ acts as an autoinducer independent of PQS. Other AQs such as NHQ can also activate PqsR and as such could potentially be considered as autoinducers, although not as potent as PQS (Xiao et al., 2006a; Fletcher et al., 2007).

The las and rhl QS systems are linked to AQ production and regulation, forming an incoherent feed-forward loop likely to produce accelerated pulse-like responses (Alon, 2007): the las system positively controls AQ production by inducing the pqsR and pqsA promoters and the rhl system downregulates its effects (Pesci et al., 1999; McKnight et al., 2000; McGrath et al., 2004; Wade et al., 2005; Xiao et al., 2006b) (Fig. 5). In a lasR mutant, transcription of pqsR is reduced about fourfold compared with the wild type (Wade et al., 2005) and LasR appears to induce pqsR transcription by binding to a conserved las/rhl box situated 517–536 bp upstream of its translational start site (McGrath et al., 2004; Xiao et al., 2006b; Gilbert et al., 2009). In line with this, a transcriptional pqsR-lacZ fusion can be significantly induced in E. coli expressing lasR by the addition of 3-oxo-C12-HSL, indicating that the LasR/3-oxo-C12–HSL system acts as an inducer of pqsR (Wade et al., 2005). A lasR mutant accumulates the HHQ series of AQs, but produces very little PQS early in growth, a consequence of LasR also positively controlling the expression of pqsH, which encodes the monoxygenase required for the conversion of HHQ to PQS (Whiteley et al., 1999; Gallagher et al., 2002; Déziel et al., 2004). The transcription of pqsA is considerably reduced in a lasI mutant (McGrath et al., 2004). However, a functional las QS system is not required for AQ biosynthesis, as a lasR mutant still produces PQS in the late stationary phase and expressions of pqsR and pqsH in a lasR mutant are delayed, but not abolished during growth (Diggle et al., 2003; Xiao et al., 2006b). As RhlR overexpressed from a plasmid partially overcomes the delay in PQS production caused by a lasR mutation in strain PA14 (Dekimpe & Déziel, 2009), it appears that RhlR could replace some of the functions of LasR with respect to the pqsA and pqsH promoters to induce the production of PQS, although this is somewhat paradoxical because RhlR is generally considered to be a repressor of AQ production and indicates that the current LasR-RhlR-AQ QS hierarchy model in P. aeruginosa may be somewhat more sophisticated than currently thought.

While the las QS system positively regulates AQ and PQS production, the rhl system acts as a negative modulator of their regulatory effects (Fig. 5). A 50% increase in pqsR transcription has been observed in an rhlR mutant, suggesting in this case that RhlR has a repressive effect (Wade et al., 2005). Similarly, transcription from the pqsA promoter is enhanced in an rhlI mutant and addition of C4-HSL to antagonize the induction of pqsA by 3-oxo-C12-HSL, with the consequence of reducing the production of PQS (McGrath et al., 2004). Two las/rhl boxes are found at 311 and 151 bp upstream of the pqsA transcriptional start site (Xiao et al., 2006b). Deletion of the distal las/rhl box in this promoter increases transcription, while additional deletion of the proximal box does not further increase pqsA promoter activity. The deletion of rhlI causes an increase in the transcription of pqsA independent of the presence of the −311 box, suggesting that RhlR binds to this box and causes a downregulation of the pqsA promoter, whose mechanism is still unclear. In vitro electrophoretic mobility shift assays carried out on a 253-bp DNA fragment containing part of the pqsA promoter using lysates of E. coli producing RhlR in the presence or absence of C4-HSL-RhlR did not indicate binding to this region; however, the fragment used did not include the las/rhl box situated 311 bp upstream of the transcriptional starting point (Wade et al., 2005). Identification of LasR targets in vivo using chromatin immunoprecipitation coupled to DNA microarray hybridization (ChIP-chip) identified this distal las/rhl box as a LasR-binding site (Gilbert et al., 2009). As the rhl system is itself driven by the production of PQS, a negative auto-regulatory feedback loop is formed (Diggle et al., 2003). The simultaneous provision of exogenous C4-HSL and PQS restores rhlI transcription levels in a lasR mutant comparable with the wild type. However, under the same conditions, the addition of these molecules separately did not cause increased rhlI transcription, suggesting a synergistic mechanism involving the two signalling molecules (McKnight et al., 2000).

Thus, in P. aeruginosa, the autoinducible AQ system is upregulated by the las and downregulated by the rhl QS systems. AQ production is furthermore indirectly self-limited by the positive regulatory effects it exerts on the rhl QS system (Fig. 5).

**Regulation of virulence factor expression by AQs**

The first demonstration that AQs regulate virulence factor production in P. aeruginosa was that PQS positively controlled the expression of the lasB (elastase) gene (Pesci et al., 1999). It was later shown that this effect was considerably
enhanced when PQS and C4-HSL acted synergistically to upregulate lasB expression (McKnight et al., 2000). The regulation of virulence factor production by AQs is not restricted to elastase. Addition of PQS upregulates the expression of lecA and pyocyanin production in a concentration-dependent manner (Diggle et al., 2003). However, PAO1 cultures growing in the presence of PQS above a concentration of 100 μM had an extended lag phase and reached reduced ODs at the stationary phase. Despite this, the expression of lecA occurred at lower population densities and therefore the maximal expression was still observed during the early stationary phase, although the addition of PQS still resulted in an advancement of lecA expression and elastase and pyocyanin production into the logarithmic phase (Diggle et al., 2003). It worth noting that these effects were not seen when either HHQ or 3-formyl-HHQ instead of PQS was added to the cultures. Previous studies found that both RhlR and RpoS are essential for lecA expression (Winzer et al., 2000) and addition of PQS failed to restore lecA transcription in rhlR or rpoS mutants, confirming the importance of these two regulators for lecA promoter activity. However, PQS was able to overcome the repression of lecA by the H-NS-type protein MvaT and the post-transcriptional regulator RsmA (Diggle et al., 2003). We have recently found that PQS, but not HHQ, induces the transcription of the small regulatory RNA RsmZ (Fig. 5), a mechanism that explains how post-transcriptional regulation by RsmA can be overcome by PQS and reveals that this

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**Fig. 5.** Regulation of AQ production in *Pseudomonas aeruginosa*. The las QS system positively regulates the transcription of pqsR, pqsABCDE and pqsH. The PqsABCD proteins synthesize HHQ, which is converted to PQS by PqsH. Autoinduction occurs when either HHQ or PQS binds to PqsR and enhances the expression of the pqs operon. The rhl QS system, also positively controlled by the las system, exerts a negative effect on the AQ system, although it is itself positively regulated by AQs. The terminal output of this regulatory network is the PqsE protein of still unknown enzymatic function. In addition, PQS, via an unknown mechanism, positively controls the transcription of the small RNA RsmZ, which in turns has a negative effect on the RNA-binding protein RsmA involved in post-transcriptional regulation. Biosynthetic enzymes are represented by globular shapes, while transcriptional regulators are shown as cubes. Filled arrows and blunted lines represent positive and negative regulation, respectively.

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S. Heeb et al.
molecule can act on the expression of virulence genes at both the transcriptional and the post-transcriptional levels (S. Heeb et al., unpublished data).

Virulence factor production is also affected when AQ production is inhibited. Addition of the anthranilate analogue, methyl-anthranilate, to *P. aeruginosa* caused a decrease in the production of PQS and a subsequent reduction in elastase produced (Calfee et al., 2001). The effects observed with methyl-anthranilate are not restricted to elastase, with concentrations of 500 μM completely inhibiting the expression of *lecA* and pyocyanin production, but with no adverse effect on growth. This effect could partially be restored by the provision of exogenous PQS (Diggle et al., 2002). This effect on growth. This effect could partially be restored by the provision of exogenous PQS (Diggle et al., 2002; Diggle et al., 2003). The subset of genes regulated by AQs has now been examined in greater detail using transcriptomic analysis. It has been found that PqsR, through the induction of the *pqsABCDE* operon and the action of PqsE, positively regulates a subset of LasR- and RhlR-dependent genes. A *pqsR* mutant of strain PA14 displayed the upregulation of 121 and the repression of 22 mRNAs when compared with the corresponding wild type (Déziel et al., 2005). In this *pqsR* mutant, the transcription of the *pqsABCDE* and *phnAB* operons was abolished and that of *pqsR* itself was reduced. The transcription of the *phzI* operon, *hcnABC*, *chiC* (chitinase), *mexGHI-opmD*, *lecA* and *lecB* was also found to be reduced in the absence of PqsR.

However, the role of AQs in the regulation of virulence gene expression is now the subject of some debate. It had been demonstrated previously that in both *pqsR* and *pqsE* mutants, pyocyanin production, *phzA1* expression, *LecA*, elastase and rhamnolipid production levels were considerably reduced compared with the wild type (Cao et al., 2001; Gallagher et al., 2002; Diggle et al., 2003; Déziel et al., 2005) and that the addition of PQS, HHQ or HQNO to these mutants could not restore these phenotypes (Gallagher et al., 2002; Diggle et al., 2003; Déziel et al., 2005). Altogther, these studies suggested that AQ production may not be indispensable for the regulation of these phenotypes. A subsequent study shed new light on the mechanisms by which AQs induce gene transcription by revealing that PqsE alone can drive the expression of the target genes, through the *rhl* QS system (Farrow et al., 2008). By expressing PqsE in AQ-negative *pqsA* or *pqsR* mutants, it was demonstrated that pyocyanin, rhamnolipid and elastase production could be restored in the absence of AQs. This restoration of exoproducst was not observed in an *rhlR* mutant, which suggests that PqsE may exert its effects through the *rhl* system (Farrow et al., 2008). These findings raise a number of intriguing questions as to the function of AQs in *P. aeruginosa*. For example, is the primary function of both, PQS and HHQ, to bind PqsR and to upregulate the *pqsABCDE* operon, thereby forming, on the one hand, an autoinduction loop and ultimately, on the other, producing as the major output, an increase in the levels of PqsE? Another intriguing question raised by the data is about the function of PQS itself. There are conflicting reports on the necessity of PQS for virulence in different *P. aeruginosa* wild-type strains, although different hosts have been used: PQS has been shown to be necessary for the virulence of strain PA01 in nematodes (Gallagher et al., 2002), but unnecessary for PA14 in a burned mouse model (Xiao et al., 2006a). There are also conflicting reports as to the efficacy of PQS at inducing the *pqsA* promoter via PqsR. One study found that in PA14, PQS was more effective than HHQ at upregulating *pqsA* (Xiao et al., 2006a), but conversely, another study demonstrated that in strain PA01, PQS was the less effective molecule (Fletcher et al., 2007). This contradiction may be due to differences in strain-specific mechanisms, but taken together with new research on the role of PqsE, PQS may not be as important to the direct regulation of virulence factors in *P. aeruginosa* as first envisioned and may have evolved as a fortuitous byproduct with other functions (Bredenbruch et al., 2006; Diggle et al., 2007). Furthermore, the primary role of PqsR requires some further clarification. It is probable that the loss of virulence noted in *pqsR* mutants (Cao et al., 2001; Déziel et al., 2005) is primarily due to the corresponding loss of PqsE production, seen in the fact that mice mortality in strain PA14 was much decreased from the wild type and was equivalent in both *pqsA* and *pqsE* mutants (Déziel et al., 2005). Therefore, the primary role of PqsR may be that it is responsible for the expression of *pqsE* via the production of AQs and the corresponding autoiniduction of the *pqsABCDE* operon, at least as far as the production of pyocyanin and expression of *lecA* are concerned. The induction of pyocyanin production by the AQ QS system further leads to the regulation of the PYO stimulon, a set of around 50 genes whose expression is affected, primarily via the transcriptional regulator SoxR, by this phenazine (Dietrich et al., 2006).

**Role of AQs in iron metabolism**

In addition to its role as a cell-to-cell signalling molecule, PQS is also able to chelate ferric iron (Fe$^{3+}$). The presence of the 3′-hydroxy group on the molecule mediates this and allows two or three PQS molecules to bind Fe$^{3+}$ at physiological pH ranges of 6–8. Compounds similar to PQS (such as C9-PQS) also possess iron-binding capabilities, but molecules lacking the 3-hydroxy group such as HHQ are unable to do so (Bredenbruch et al., 2005; Diggle et al., 2007). Addition of PQS to *P. aeruginosa* cultures upregulates the genes involved in the production of the siderophores pyoverdine and pyochelin, which are produced in response to iron starvation, as indicated by the upregulation of siderophore-mediated iron transport systems such as the...
pyochelin biosynthetic clusters (pchDCBA and pchEGF), the iron pyochelin outer-membrane receptor fpfA and the pyoverdine genes pvdE and pvdS (Bredenbruch et al., 2005; Diggle et al., 2007). The pch genes were upregulated at 5, 11 and 20 h after inoculation between 3- and 25-fold. Also, the genes pvdJAD encoding pyoverdine synthetases were upregulated between 2- and 10-fold at 11 and 20 h (Bredenbruch et al., 2005). Quantitative real-time PCR showed that pvdA and pchE are upregulated by 6- and 17-fold, respectively, upon addition of 20 μM PQS (Diggle et al., 2007). Also, in strain PAO1 wild type as well as in pqsA, pqsE or pqsR mutants, the addition of PQS, but not of HHQ, strongly induced pyoverdine production (Diggle et al., 2007).

PQS, with its effect on free iron levels, also affects the transcription of other genes. During growth in iron-replete media, both lecA and pqsA were strongly induced by the addition of 50 μM PQS in a PAO1 pqsA mutant. However, the induction of pqsA was not due to the iron-chelating properties of PQS because when grown in an iron-deficient casamino acid (CAA) medium, PQS, PQS–Fe3+(3:1) and HHQ all induced the pqsA promoter, but methyl-PQS did not (Diggle et al., 2007).

Around 60% of the PQS produced by P. aeruginosa is associated with the cell envelope (Lépine et al., 2003; Diggle et al., 2007), and the membranes of cells grown in iron-rich media are visibly pink due to complexed Fe3+, possibly stored in AQ-containing inclusion bodies (Royt et al., 2001, 2007). Therefore, there is the possibility that PQS could act as an iron trap and storage molecule in the cell membrane and that it may be able to deliver iron directly to the cells. However, experiments carried out with a P. aeruginosa pvdD/pchEF double mutant, which lacks any iron acquisition systems, revealed that it was unable to grow in an iron-deficient CAA medium in the presence of added PQS. In contrast, this mutant had a similar growth compared with the parental PAO1 strain when exogenous PQS was not added to the medium. These data suggest that although PQS may trap iron in the cell membrane, it is unlikely that it can act as a siderophore per se (Diggle et al., 2007).

Iron-dependent regulation of AQ production appears to be controlled by the availability of one of their precursors, anthranilate. Under iron-limiting conditions, the ferric uptake regulator Fur does not repress the transcription of two genes pprF1 and pprF2, encoding small regulatory RNAs (Wilderman et al., 2004), which post-transcriptionally repress the expression of the antABC and catBCA operons specifying enzymes for the degradation of anthranilate. Hence, in a pprF1 pprF2 double mutant, PQS production is abolished under iron-limiting conditions, probably as a consequence of anthranilate depletion (Oglesby et al., 2008). Therefore, under iron-limiting conditions, the supply of anthranilate for the biosynthesis of AQS is controlled by Fur and the PrrF sRNAs, an effect that was further reinforced by the iron starvation response resulting from the iron-chelating property of PQS (Bredenbruch et al., 2006; Diggle et al., 2007).

Because HHQ performs functions similar to those of PQS, such as the induction of the pqsA promoter (Xiao et al., 2006a; Diggle et al., 2007), many of the specific effects observed upon addition of PQS may be due to its iron-chelating properties. It is also probable that the production of PQS and its chelating effects could confer a survival advantage when P. aeruginosa is growing with other competing microorganisms in iron-limited environments. The red-coloured PQS–Fe3+ complex can also be toxic to other organisms. For example, its production has been found to confer the 'red death' lethal phenotype to P. aeruginosa in a Caenorhabditis elegans infection model (Zaborin et al., 2009).

Iron availability also influences the levels at which AQS induce the activity of PqsR as a transcriptional activator, and therefore, iron also acts directly as a modulator of the AQ signalling system in P. aeruginosa (Hazan et al., 2010). Interestingly, iron has also been found bound to PqsE, although without knowledge of the function of this enzyme, the biological significance of this remains unclear (Yu et al., 2009).

Additional regulators of AQ production in P. aeruginosa

Besides autoinduction by PQS and its precursor HHQ, modulation by the las and rhl QS systems, and metabolic and regulatory adjustments following iron availability, AQ production is regulated by additional factors (Table 1). For example, AQ production is enhanced under phosphate-limiting conditions. In P. aeruginosa, the transcriptional regulator PhoB mediates responses to phosphate limitation (Anba et al., 1990). As a PHO box has been found overlapping the distal transcriptional starting point of pqsR and as AQ production is no longer enhanced in a phoB mutant, these elements may mediate the increased AQ production observed following phosphate limitation (Jensen et al., 2006).

PtxR is a transcriptional regulator that positively affects the production of exotoxin A and negatively affects the production of pyocyanin. PtxR reduced the expression of the pqsABCDE operon, probably indirectly and not via the repression of pqsR (Carty et al., 2006). However, PtxR also regulated the las positively and the rhl QS systems negatively, which paradoxically should have resulted in an induction of the pqsA promoter. Therefore, it appears that PtxR could be part of an intricate network of feed-forward loops (Alon, 2007) that connect the las, rhl and pqs QS systems.

The gene mpnR (pqsR-mediated PQS regulator, PA0964) was found by screening transposon mutants for clones in...
which the phzA1 promoter had altered expression profiles. PmpR, a protein of the YebC-like superfamily, binds to the pqsR promoter and affects its transcription negatively. A pmpR mutant was therefore found to have increased mRNA levels of pqsR, pqsA and pqsH, which was suggested to result in the observed induction of the phzA1 promoter and of pyocyanin production, and enhanced swarming motility and biofilm formation (Liang et al., 2008).

The gene ppyR (psl and pyoverdine operon regulator, PA2663) appears to encode a membrane sensor that positively regulates exopolysaccharide and pyoverdine production, perhaps in response to the presence of NOR (Attila et al., 2008). The deletion of ppyR caused the downregulation of several genes including the pqsABCDE operon and the pqsH gene for AQ and PQS biosynthesis, as well as the antABC operon for anthranilate degradation. As a consequence, a ppyR mutant produced no detectable PQS. However, the mechanism by which PpyR exerts its effects or the signals that it senses are unknown.

### Impact of AQs on microbial interactions

The prominence of *P. aeruginosa* as a major opportunistic pathogen in nosocomial infections and in lung infections in...
CF patients (Govan & Deretic, 1996) led to the investigation of the role of AQs in the regulation of virulence factor production and the establishment and severity of infection. The initial studies using clinical isolates from sputum in CF patients showed that they all produced PQS (Collier et al., 2002) and also HHQ, HQNO, NQNO and UQNO (Machan et al., 1992). In addition, there was a correlation between the levels of PQS and the bacterial sample load. Furthermore, PQS was also found in isolates from paediatric CF patients and from patients at early stages of P. aeruginosa infection (Guina et al., 2003). The regulation of PQS production in some of these isolates was irregular as this molecule was detected early in growth, during the log phase. The use of a simulated CF sputum medium has been shown to support the growth of P. aeruginosa to high population densities (Palmer et al., 2005) and also the differential regulation of PQS production. In particular, the expression of the phnAB genes is induced 14–22-fold, in line with the upregulation of the pqsABCDE operon (17–19-fold) compared with the expression of these genes in a morpholinepropanesulfonic acid-buffered glucose medium. This upregulation results in a fivefold increase in PQS production and presumably the other AQs, and is not triggered by changes in AHL levels. It is possibly linked to the presence of aromatic amino acids in the sputum medium such as tryptophan, which is used by P. aeruginosa for anthranilate biosynthesis (Farrow & Pesci, 2007). A recent study revealed that although PhhR is an aromatic amino acid-responsive transcriptional regulator that controls genes involved in phenylalanine and tyrosine catabolism in P. aeruginosa, the biosynthetic genes for AQs are not differentially expressed according to the presence of this regulator (Palmer et al., 2010).

Pseudomonas aeruginosa forms biofilms to protect itself from the harsh environmental conditions generated by the host immune system and antimicrobials. AQs play an important role in the establishment and maintenance of the biofilm lifestyle by a number of different mechanisms. The exogenous addition of 60 μM PQS to growing cultures of P. aeruginosa PAO1 resulted in a significant enhancement in biofilm formation partly due to the induction of expression of the lectin gene lecA (Diggle et al., 2003) as this gene plays a role in maintaining biofilm architecture in this organism (Diggle et al., 2006b). Pseudomonas aeruginosa can also release, possibly through lysis of cell subpopulations, extracellular DNA, which acts as an interconnecting matrix in bacterial biofilms (Whitchurch et al., 2002). DNA has cation-chelating and antimicrobial properties and can cause the disruption of the bacterial outer membrane by chelating Mg$^{2+}$, which is essential for membrane stability. This in turn could result in more DNA release (Mulcahy et al., 2008). In addition, Mg$^{2+}$ chelation induces the expression of the PhoPQ two-component system, increasing the resistance of P. aeruginosa towards aminoglycosides such as gentamicin and cationic antimicrobial peptides. These broad-spectrum antimicrobial peptides are released from host immune cells and can disrupt the bacterial outer membrane, causing cell death. Maximum DNA release takes place in the late log phase when PQS production is at its highest (Diggle et al., 2003; Lépine et al., 2003). Similarly, a pqsA mutant releases low levels of extracellular DNA and forms flat, thin unstructured biofilms with increased sensitivity to detergents. The detergent sensitivity may be due to the loss of this extracellular DNA as a wild-type biofilm treated with DNase retains this sensitivity (Allesen-Holm et al., 2006; Haussler & Becker, 2008). A correlation between bacterial cell lysis and PQS levels has been established, which may explain the release of the extracellular DNA observed in biofilms (D’Argenio et al., 2002). A mutation in the pqsL gene (which results in PQS overproduction) resulted in pronounced lysis in bacterial colonies, whereas those from pqsA and pqsR mutants displayed no lysis, but this could be restored upon addition of exogenous PQS. It has been proposed that PQS induces prophage-mediated lysis and that this is responsible for the DNA release (D’Argenio et al., 2002). The chromosome of P. aeruginosa harbours the filamentous Pf4 prophage, whose deletion results in the loss of bacterial autolysis and aberrant biofilm formation (Rice et al., 2009). PQS also acts as a pro-oxidant, which can increase the sensitivity of P. aeruginosa to peroxide and ciprofloxacin (Häußler & Becker, 2008), possibly resulting in cell lysis and DNA release.

AQs inhibit the growth of S. aureus and the yeast C. albicans, suggesting that they may be used as antibiotics by P. aeruginosa, during the early stages of infection, enabling it to eradicate any competing organisms (Machan et al., 1992). This idea is further supported by the fact that AQs packaged in MVs inhibited the growth of S. epidermidis (Mashburn & Whiteley, 2005), whereas mutants in kynAU were unable to kill S. aureus and a kynB mutant displayed reduced killing, presumably due to the lack of AQ production (Farrow & Pesci, 2007). As mentioned earlier (Natural antimicrobial quinolones), both HHQ and PHQ have antibacterial activities, while PHQ additionally presents antialgal properties (Wratten et al., 1977; Long et al., 2003). HHQ and HQNO produced by a clinical isolate of P. aeruginosa inhibited the growth of metronidazole-resistant H. pylori in a cross-streak assay (Lacey et al., 1995). These findings may explain why early colonizers of the CF lung such as S. aureus are sometimes absent upon P. aeruginosa colonization, which outcompetes other organisms sharing the same niche (Machan et al., 1992). Consequently, the combined iron-chelating properties and the impact on virulence factor production of AQs help P. aeruginosa to generate a highly favourable environment in which to thrive.

Interestingly, farnesol, a sesquiterpene signal molecule produced by C. albicans, reduces the transcription of pqsA
by interacting with PqsR and probably interfering with the normal binding of this transcriptional regulator to the \( pqsA \) promoter (Cugini et al., 2007). This results in a decrease in both PQS and pyocyanin production and suggests that this type of interspecies competition can be reciprocal. In addition, HQNO has been shown to induce the formation of persistent small-colony variants of \( S.\ aerues \) that may resist \( P.\ aeruginosa \) niche colonization and possibly explain the coexistence of these two organisms in some infections (Hoffman et al., 2006).

**Roles of AQs in infection**

The role of AQs in virulence and the severity of infection has been demonstrated using several disease models. A mutation in \( phnAB \) resulted in a fourfold decrease in virulence compared with the wild type in a wax moth (\( Galleria mellonella \)) larvae model (Jander et al., 2000). In addition, mutations in \( pqsC, pqsD, pqsE, pqsR, pqsH \) and \( phnA \) resulted in severely reduced killing of the nematode (\( C.\ elegans \)) by \( P.\ aeruginosa \) to between 37 and 39% of the wild-type levels (Gallagher et al., 2002). Using a burned mouse model, mutants in \( pqsA \) and \( pqsE \) also exhibit reduced virulence (Déziel et al., 2005; Rampioni et al., 2010). In the same disease model, a \( pqsR \) mutant showed an \~{}35% reduced mortality rate compared with the wild type. This mutant also showed reduced PQS, 3-oxo-C12-HSL, pyocyanin, elastase and exoprotein production (Cao et al., 2001). Most interestingly, a \( pqsH \) mutation in \( P.\ aeruginosa \) PA14 was not attenuated, suggesting that PQS may not be essential for virulence and that virulence may be regulated via the biosynthetic precursor, HHQ (Xiao et al., 2006a).

Virulence factor and AQ production are upregulated in response to host stress responses to \( P.\ aeruginosa \). The synthetic opioid U-50,488 and the endogenous \( \kappa \)-opioid receptor agonist dynorphin, which is released into the human small intestine during inflammation and appears to bind and enter bacterial cells, have been tested in \( P.\ aeruginosa \) PAO1 and were found to enhance virulence factor production (Zaborina et al., 2007). Furthermore, the addition of U-50,488 or dynorphin to a PAO1 culture induced a dose-dependent increase in pyocyanin production and enhanced \( paqA \) and \( locA \) (but not \( pqsR \) expression), with a corresponding increase in PQS, HHQ and HQNO production. These opioid agonists also enhanced \( P.\ aeruginosa \) virulence against \( Lactobacillus \) and \( C.\ elegans \), probably as a result of the above increases in virulence determinant production.

AQs may also interfere with host responses by acting as immune modulators. PQS suppresses T-cell proliferation and interleukin-2 release in concanavalin A-activated human peripheral blood mononuclear cells (hPBMCs). PQS also induces tumour necrosis factor-\( \alpha \) release from lipopolysaccharide-activated hPBMCs, at concentrations around 10 \( \mu \)M (Hooi et al., 2004). *In vitro*, PQS reduces the release of interleukin-12 from lipopolysaccharide-stimulated bone marrow-derived dendritic cells, preventing the development of naïve T cells into T-helper type 1 cells, which promote cell-mediated immunity. The concentration of PQS required to lower the cytokine release to 50% in this case was below 20 \( \mu \)M (Skindersoe et al., 2009). Additionally, both HHQ and PQS appear to suppress host innate immune systems by interfering with the nuclear transcription factor-kB signalling pathway. This effect can be achieved with cell-free extracts from cultures of wild-type \( P.\ aeruginosa \), but not from the cultures of a corresponding \( pqsA \) mutant (Kim et al., 2010). It therefore seems possible that AQs play a role in the dysregulation of the host immune response during infection.

**Production of AQs by other bacteria**

DNA database analysis has revealed the presence of \( pqs \) gene homologues in \( > 40 \) species and strains that are more or less related to \( P.\ aeruginosa \). In particular, *Burkholderia pseudomallei* and *B. thailandensis* appear to have the complete putative \( pqsABCDE \) operons in their chromosomes, sharing 31–53% identity to that of *P. aeruginosa* (Diggle et al., 2006a). These were named \( hhqABCDE \) as no PQS had been detected in these organisms. The \( hhqA \) and \( hhqE \) genes are functionally conserved with their *P. aeruginosa* homologues as they were able to complement PAO1 \( pqsA \) and \( pqsE \) mutants, respectively, and restore PQS, HHQ, lectin and pyocyanin production in the \( pqsA \) mutant and pyocyanin and lectin production in the \( pqsE \) mutant. Using a combination of a novel AQ bioreporter and LCMS/MS, HHQ was detected in culture supernatants of *Pseudomonas putida* and *Burkholderia cenocepacia* and HHQ, NHQ, UHQ and HQNO in *B. pseudomallei* (Diggle et al., 2003). Although a mutant unable to generate AQs in *B. pseudomallei* presented altered colony morphology and increased elastase production, the actual role of these molecules in the biology of this organism remains to be unravelled. AQs have also been identified in a number of species of *Burkholderia* such as *Burkholderia ambifaria*, *B. thailandensis*, *B. pseudomallei* and *Pseudomonas cepacia* (probably an unclassified *Burkholderia*). The main AQs produced by these organisms are 3-methyl derivatives of PHQ, HHQ and NHQ termed 4-hydroxy-3-methyl-2-alkylquinolines (Moon et al., 1996; Vial et al., 2008). Consequently, the operon responsible for their synthesis has been renamed \( hhqABCDEFG \) (formerly \( hhqABCDE \)), with the predicted methyltransferase \( hhqG \) being involved in the biosynthesis of these AQs. None of the above bacteria has \( pqsH \) orthologues nor produces PQS, and previous efforts to detect PQS in other pseudomonads...
such as _P. fluorescens_, _Pseudomonas syringae_ and _Pseudomonas fragi_ have been unsuccessful (Lépine _et al._, 2004). Furthermore, in _B. thailandensis_, _B. ambifaria_ and _B. pseudomallei_, the –3′ position is largely methylated (Vial _et al._, 2008), which would presumably preclude any _pqsH_ analogue hydroxylating in these molecules and hence the production of PQS. These findings suggest that these organisms may lack the complexity of PQS signalling found in _P. aeruginosa_.

**Concluding remarks**

The discovery of quinine and related natural antiplasmodial alkaloids, combined with the advances of synthetic chemistry, spurred significant research in the field and resulted in the development and evaluation of thousands of novel synthetic compounds. Among these were naldixic acid and the extensive family of synthetic quinolone antibiotics. In parallel, the quest for antimicrobials of natural origin lead to the discovery of the AQNOs and of the extensive family of AQ compounds mostly produced by _P. aeruginosa_ and related bacteria. Synthetic and natural quinolone antimicrobials, however, appear to share little in common with respect to their mode of action. The biological roles of the natural quinolones of bacterial origin are diverse and include intercellular signalling. The discovery of a non-AHL-based QS system in _P. aeruginosa_ mediated via AQs and linked to the _las_ and _rhl_ QS systems provides a major insight into a complex regulatory network that plays key roles in infection via the regulation of virulence and biofilm maturation. Some AQs, such as PQS, are also able to sequester iron and have multiple functionalities. AQ biosynthesis requires several proteins and occurs via a condensation reaction between anthranilate and ß-keto fatty acids. Their production is upregulated by both the _las_ QS system and by AQs themselves and downregulated by the _rhl_ QS system. AQs are present in bacteria other than _P. aeruginosa_, mainly other pseudomonads and _Burkholderiaceae_, although the role in these organisms is not, at present, very well defined. It is possible that AQs could be produced by many other bacterial genera, as the number of studies where these compounds have been specifically screened has been small and yet several AQs-producing species have been discovered (Lépine _et al._, 2004; Diggle _et al._, 2006a; Vial _et al._, 2008). In most of the species that produce AQs, the role of these compounds or their biosynthesis remains unclear, although for many where genomic sequences are available, orthologues of the _pqsABCDE_ operon extensively studied in _P. aeruginosa_ can often be identified.

Quorum quenching is the process by which the signalling mediating QS is interfered with, leading to the disruption of the normal means by which bacteria coordinate their behaviour according to their population density and preventing colonization (Raina _et al._, 2009). Quorum quenching can be exerted naturally by microorganisms to prevent the establishment of competing species and offers a strategy for the development of novel antimicrobial drugs (Rasmussen & Givskov, 2006). Hence, quinolone quenching offers the possibility to interfere with AQ signalling in pathogens such as _P. aeruginosa_, which rely on it to control virulence. As a primary target to interfere with AQ-mediated signalling, binding of PQS and HHQ to PqsR could be blocked. This would not only interfere with the production of AQs and their associated properties beneficial for the bacterial cell by preventing the expression of the _pqsABCDE_ operon, but would also downregulate all the other AQ-regulated genes, including those essential for virulence. Compounds such as farnesol inhibit the induction capacity of PqsR on the _pqsA_ promoter (Cugini _et al._, 2007). However, as these compounds were found to act only at relatively high concentrations (in the mM range), other, more potent inhibitors are needed to stimulate clinical interest. Another possibility would be to inhibit the action of PqsE, whose function is still unclear, but that is required for the production of several virulence factors. Analogues of AQ precursors such as methyl-anthranilate or halogenated derivatives of anthranilate have been found to inhibit AQ synthesis, thus interfering with the signalling system probably by acting as competitive inhibitors of PqsA (Calfee _et al._, 2001; Lesic _et al._, 2007; Coleman _et al._, 2008). This approach has recently shown promising results in limiting the systemic proliferation of _P. aeruginosa_ infection in mice (Lesic _et al._, 2007).

To complete our understanding of the AQ signalling system in _P. aeruginosa_, the roles of some components still remain to be fully unravelled. PqsB, PqsC and PqsL are likely to be involved in the biosynthesis of AQs as revealed by the structural domains they share with other known enzymes and by mutational analysis (Gallagher _et al._, 2002; Diggle _et al._, 2003; Breidenbruch _et al._, 2005; Farrow _et al._, 2008). However, the exact role of PqsB and PqsC in the biosynthesis of HHQ and PQS still remains unclear. Similarly, little is known about the interaction of PqsL with precursor and other biosynthetic proteins to generate the _N_-oxide AQNOs, because the otherwise common precursor molecule HHQ does not appear to be required for their production (Déziel _et al._, 2004). Even more critically, the role played by PqsE, which appears to be an enzyme of the metallo-β-hydrolase superfamily mediating the signal transduction that upregulates swarming motility, the production of pyocyanin, lectin, HCN, the transcription of many genes and ultimately virulence (Rampioni _et al._, 2010) remain to be elucidated.

The biology of AQ biosynthesis, its regulation and the signalling functions of the AQs control the behaviour and virulence of _P. aeruginosa_. AQ signalling is proving to be complex, leading to many open questions that still remain unanswered. For example, the biological roles of AQs such...
as DHQ or the N-oxide derivatives in P. aeruginosa remain to be elucidated, as are the functions of AQS in other pathogenic and beneficial microorganisms producing them. From their inconspicuous discovery as potentially useful compounds having weak antimicrobial properties, quinolones in general and AQS in particular are highly versatile molecules that play central roles in the biology of the producer organisms.

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