Structure-Activity Analysis of the *Pseudomonas* Quinolone Signal Molecule[▽]

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We synthesized a range of PQS (*Pseudomonas* quinolone signal; 2-heptyl-3-hydroxy-4(1*H*)-quinolone) ana-logues and tested them for their ability to stimulate MvfR-dependent *pqsA* transcription, MvfR-independent pyoverdine production, and membrane vesicle production. The structure-activity profile of the PQS analogues was different for each of these phenotypes. Certain inactive PQS analogues were also found to strongly synergize PQS-dependent pyoverdine production.

Pseudomonas aeruginosa is a major cause of chronic infec-tions, especially among individuals with cystic fibrosis (CF) (12, 14). Virulence factor and biofilm production by *P. aeruginosa* is controlled by quorum sensing (QS). The QS system of *P. aeruginosa* involves the following two distinct types of signaling molecules: *N*-acylated homoserine lactones (AHLs) (23) and the *Pseudomonas* quinolone signal (PQS; a 4-alkyl-quinolone) (18). The biosynthetic precursor of PQS, 2-heptyl-4(1*H*)-quin-olone (HHQ), has also been demonstrated to function as a signaling agent in *P. aeruginosa* (24). AHL-dependent signaling is widespread among Gram-negative bacteria (19). However, 2-alkyl-4-quinolone (AHQ) signaling is more restricted and has so far been detected only in *P. aeruginosa* and certain *Burkholderia* and *Alteromonas* spp. (7, 21). PQS has been re-ported in the sputa of CF patients (estimated minimal concen-tration of ca. 2 μM) (3), indicating that it may be an important factor in the establishment of chronic infections.

PQS has been shown to control production of multiple vir-ulence determinants, including elastase, pyocyanin, rhamnolip-ids, and the siderophore pyoverdine (5, 9, 11, 18). PQS is extremely hydrophobic and is trafficked between cells in mem-brane vesicles (MVs) (17). MV formation is also stimulated by PQS, which has been shown to interact with the lipopolysac-charide (LPS) layer of the outer membrane (16). PQS biosyn-thesis is directed in 2 steps. The PqsABCD proteins catalyze the condensation of β-keto fatty acids with anthranilate to yield AHQs, prominent among which is 2-heptyl-4(1*H*)-quin-olone (HHQ) (2, 6, 18). HHQ is a diffusible molecule that can be produced in one cell and then converted to PQS in another through the action of an unlinked monooxygenase, PqsH (6, 8). Synthesis of PQS is primarily under the control of the AHL-dependent QS system (22), although it can also be produced independently of it (9, 25). LasR stimulates transcription of *pqsH* and *mvfR* (a LysR-type regulator, also known as PqsR) (5, 9, 11, 24). MvfR activates transcription of *pqsABCDE*. PQS

is not the only alkyl-quinolone made by *P. aeruginosa* (6, 18). Others include HHQ, 2-nonyl-4-quinolone (NHQ), and 2-hep-tyl-4-quinolone *N*-oxide (HHQNO). The signaling function(s), if any, of NHQ and HHQNO is not clear.

In spite of the important role(s) played by PQS in virulence, it has not yet been the subject of a systematic structure-activity relationship (SAR) analysis. Previous structural modifications of 4-alkyl quinolones have focused mostly on assessing the activity of alkyl chain congeners in stimulating transcription from the *pqsA* and *lecA* promoters (10) and MV production (15). In the current work, we generated a more diverse range of PQS analogues and tested them for their ability to stimulate (i) MvfR-dependent transcription of *pqsA*, (ii) MvfR-indepen-dent production of pyoverdine, and (iii) membrane vesicle formation. The analogues we made are shown in Fig. 1. The synthesis of these analogues will be described elsewhere (J. Hodgkinson, M. Welch, and D. R. Spring, unpublished data).

The ability of the analogues to stimulate MvfR-dependent transcription from the *pqsA* promoter was assessed as previ-ously described (4) in *Escherichia coli* strain DH5α containing pEAL08-2. This plasmid encodes MvfR (under the control of the *tac* promoter) and carries *lacZ* under the control of the *pqsA* promoter. Each analogue (or, as a control, dimethyl sul-foxide [DMSO]) was added at the start of the growth curve, and the β-galactosidase activity of the cells was measured after 8 h, as described previously (4). The results are shown in Fig. 2. In this heterologous *E. coli*-based reporter system, the con-centration of PQS required to stimulate PqsR-dependent *pqsA* transcription was ~1,000-fold lower (nM versus μM) than the concentration of PQS reported by Fletcher et al. (10) to do the same in *P. aeruginosa*. This suggests that in *P. aeruginosa*, additional factors (e.g., PQS efflux) may influence the mea-sured response and that the *E. coli*-based system represents a more direct assay of PqsR activity. There was a strong alkyl chain length dependency on agonism; analogue 1, which car-ries only a short "stub" of an alkyl chain, was essentially inac-tive, while analogues 2 and 3 (containing alkyl chains with 5 and 6 carbon atoms, respectively) displayed progressively greater stimulatory activity. Analogues with alkyl chains longer than 7 carbon atoms retained substantial agonist activity, indi-
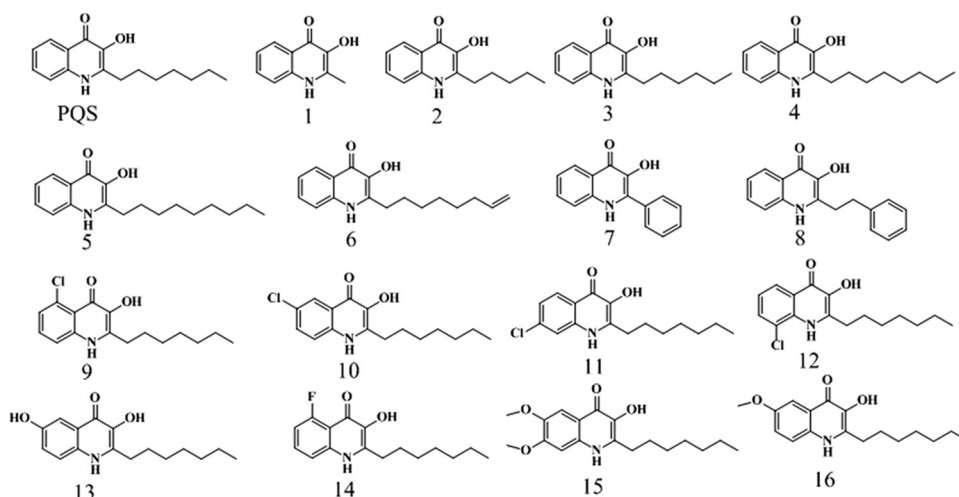
FIG. 1. Structures of the PQS analogues used in this work.

cating that the PQS-binding pocket of MvfR can accommodate these variants. These data are consistent with those of Fletcher et al. (10), who noted similar alkyl chain length dependency using a *pqsA*::*lux* reporter system in a *P. aeruginosa* PAO1 background. However, the alkyl chain binding pocket of MvfR was apparently intolerant of nonalkyl substituents, since replacement of the PQS heptyl side chain with phenyl groups (Fig. 2, analogues 7 and 8) greatly reduced or abolished agonist activity. Introduction of chlorine atoms at each of the four available positions on the anthranilate ring (Fig. 2, analogues 9 to 12) led to a rather uniform diminution in agonist activity, whereas introduction of a fluorine atom at position 5 had no effect (cf. analogues 9 and 14). This may be due to steric effects of the substituents (F has a smaller van der Waals radius than Cl), although the introduction of electron donating −OH or −OMe groups at position 6 (Fig. 2, analogues 13 and 16) or 7
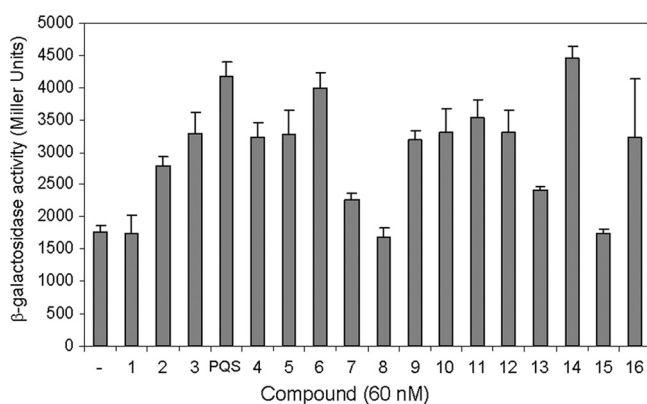

FIG. 2. Stimulation of MvfR-dependent *pqsA* promoter activity by the PQS analogues. Cultures of *E. coli* strain DH5α containing pEAL08-2 were grown in LB with good aeration at 37°C using shake flasks. Each culture contained 60 nM PQS analogue, and the β-galactosidase activity (corrected for the culture optical density at 600 nm [OD_{600}]) was measured after 2.5 h, essentially as described by Cugini et al. (4). No effect on growth was observed for any of the analogues. DMSO (−) was added as a control. The data represent the averages and standard deviations from the results of 3 independent biological repeats.

(analogue 15) led to a large reduction in activity, suggesting that the electron density of the aromatic ring may also play a part.

PQS can stimulate pyoverdine production in a MvfR-independent manner (1, 8). Since PQS is also an iron chelator, presumably due to the presence of the 3-hydroxy-pyridin-4-one moiety, this has led to the suggestion that PQS stimulates pyoverdine production indirectly by depleting the culture of utilizable iron (1, 8, 26). However, the dependence of pyoverdine production on PQS structure has not so far been systematically studied. Pyoverdine production was measured in *P. aeruginosa* strain DH125 (a PA14-derived *pqsB*::Tn*phoA* strain) (4). Due to the disruption of *pqsB*, this strain is unable to generate endogenous alkyl quinolones. Dose-response curves showed that addition of 20 to 30 μM exogenous PQS stimulated a sharp increase in pyoverdine production by DH125, which saturated at around 60 μM PQS (data not shown). Therefore, we tested the PQS analogues at 30, 60, and 90 μM concentrations (Fig. 3). Since all our analogues contain the 3-hydroxy-pyridin-4-one moiety, we expected to see relatively little difference in their abilities to stimulate pyoverdine production. However, this was not the case. At 30 μM, only analogues PQS, 4, and 6 (and to a lesser extent, analogue 3) stimulated pyoverdine production to significant levels. Also, and unlike MvfR-dependent *pqsA* transcription, there was an abrupt abolition of pyoverdine-stimulatory activity when the alkyl chain exceeded 8 carbon atoms in length (Fig. 3, cf. analogues 4 and 5). Notably, introduction of an unsaturated bond at the end of the alkyl chain (Fig. 3, analogue 6) increased pyoverdine production compared with that of the saturated equivalog (analogue 4) and PQS. Substitution of the alkyl chain with a phenyl moiety (Fig. 3, analogues 7 and 8) essentially abolished activity. In contrast to the MvfR-dependent activity profile, introduction of a fluorine atom at anthranilate ring position 5 resulted in lower agonist activity, while chlorine atom substitutions at positions 5 to 8 displayed the following activity profile: position 5 > position 6 > position 7 > position 8. Modifications that introduced electron-donating groups (Fig. 3, analogues 13, 15, and 16) were inactive at all
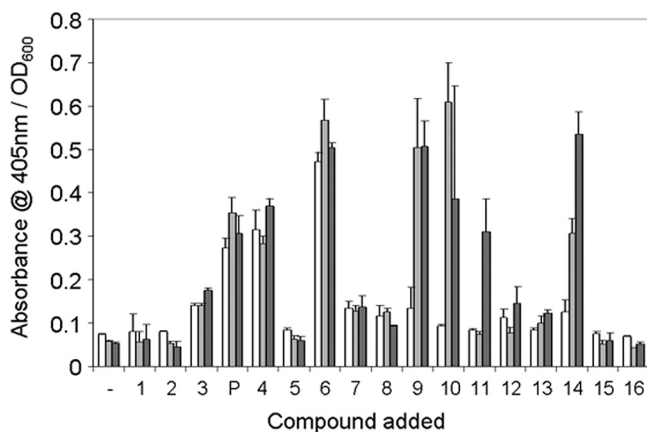
FIG. 3. Stimulation of pyoverdine production by the PQS analogues. An overnight culture of *P. aeruginosa* strain DH125 was used to inoculate LB to a starting OD_{600} of 0.05. The PQS analogues (or, as a control, DMSO) were added to a final concentration of 30 μM (white bars), 60 μM (light gray bars), or 90 μM (dark gray bars), and the samples were grown for 8 h with good aeration at 37°C. After this, the OD_{600} was recorded, and the samples were clarified by centrifugation (10,000 × *g*, 10 min, 20°C). Pyoverdine in the supernatant was quantified as previously described (8) at 405 nm. The data represent the averages and standard deviations from the results of 3 independent biological repeats. None of the analogues affected growth at any of the concentrations tested. P, PQS; −, DMSO control.
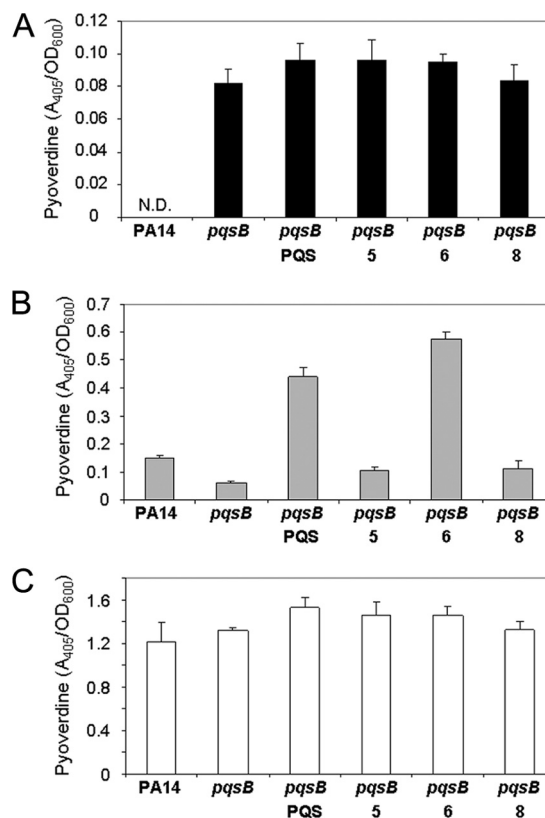

FIG. 4. Effect of iron availability on AHQ-dependent pyoverdine production. Cultures were grown, and pyoverdine was assayed as described in the legend to Fig. 3, except that the indicated AHQs were present at a 90 μM concentration and the medium used was LB supplemented with 300 μM $FeCl_3$ (A), LB alone (B), or deferrated LB (C). ND, not determined. The data represent the averages and standard deviations from the results of 3 independent biological repeats.

concentrations tested. We conclude that the stimulation of pyoverdine production shows a strong dependence on several aspects of PQS structure unrelated to the 3-hydroxy-pyridin-4-one moiety *per se*. In this regard, we note that analogue 1, which has been experimentally demonstrated to bind Fe^{3+} with high affinity (log β_3 = 36.2) (8), was unable to induce pyoverdine production at any of the concentrations tested.

To further investigate the interplay between iron availability and AHQs, we examined whether iron deprivation or iron supplementation affected the pyoverdine-inducing activity of a selection of analogues (PQS and compounds 5, 6, and 8, all at a concentration of 90 μM). As shown in Fig. 4, in the presence of excess iron in the medium (Fig. 4A), pyoverdine production was low and unaffected by the addition of the AHQs. In LB alone (Fig. 4B), the same pattern of induction as that shown in Fig. 3 was observed. In deferrated medium (Fig. 4C), pyoverdine production was uniformly high and unaffected by the AHQs. These data show that when iron is either limiting or in excess, it overrides the effect(s) of AHQs on pyoverdine production.

We wondered whether any of the "inactive" or partially active analogues might antagonize pyoverdine production. DH125 was grown in the presence of 25 μM PQS and 75 μM selected analogues (1, 2, 3, 5, 7, 8, 12, 13, 15, and 16), and pyoverdine was assayed. The results are shown in Fig. 5. No antagonism was observed. Instead, we noted that some of the compounds strongly synergized the activity of PQS. Of particular note, analogues 2 and 5, which were themselves completely inactive at stimulating pyoverdine, and analogue 12, which very poorly stimulated siderophore production, yielded substantial induction ratios (Fig. 5). Compounds 15 and 16, which were also completely inactive (Fig. 3), displayed partial PQS synergism. Only compounds 1, 7, and 8 did not display

synergism. These data raise the possibility that the nominally "nonactive" endogenous PQS analogues such as NHQ may function to "fine-tune" the activity of PQS itself. Speculatively, the molecular basis for the observed synergism might be through facilitation of PQS uptake or trafficking, perhaps linked to the ability of synergizing compounds to specifically interact with the cell envelope.

We next investigated whether the PQS analogues influenced membrane vesicle (MV) formation by *P. aeruginosa*. The results are shown in Fig. 6. Consistent with previous reports (15, 17), we found that wild-type cultures produced more MVs than the PQS null mutant. However, in our hands, most of the compounds tested (including PQS itself) were able only to slightly stimulate MV production (<2-fold) when present at a 50 μM concentration. This contrasts with the findings of earlier works that reported nearly complete restoration of MV production at this concentration of PQS (Fig. 6, wild type [WT]) (17) and may reflect the different approaches used to quantify the MVs (total protein content measured in the previous work versus phospholipid content measured in the current work). This notwithstanding, compounds 9, 10, and 11 were able to restore MV production to much higher levels than PQS, indicating that the introduction of chlorine atoms at positions 5, 6, and 7 (respectively) of the anthranilate ring increases MV-
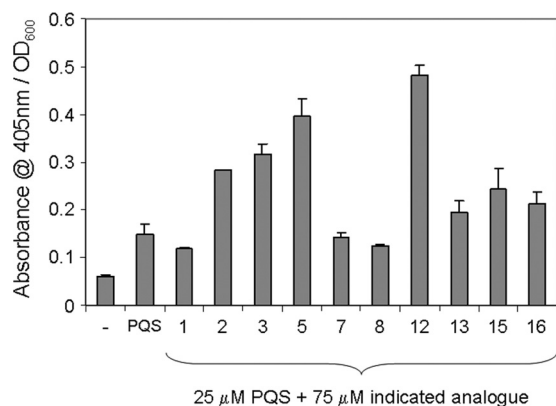
FIG. 5. Nonactive PQS analogues synergize PQS-dependent pyoverdine stimulation. Cultures of *P. aeruginosa* strain DH125 were incubated for 8 h in the absence of PQS (−), with 25 μM PQS (PQS), or with 25 μM PQS and 75 μM indicated analogues. Pyoverdine was then assayed in the culture supernatant at 405 nm (8). The data represent the averages and standard deviations from the results of 3 independent biological repeats.

TABLE 1. Lipophilicity and aqueous solubility of the PQS analogues

Analogue	Lipophilicity (logP)	Water solubility[a]
1	0.802	Soluble
2	2.942	Sparingly
3	3.447	Insoluble
PQS	3.952	Insoluble
4	4.458	Insoluble
5	4.963	Insoluble
6	3.938	Insoluble
7	2.803	Soluble
8	2.254	Soluble
9	5.425	Insoluble
10	4.606	Insoluble
11	4.606	Insoluble
12	4.582	Insoluble
13	3.449	Insoluble
14	4.911	Insoluble
15	3.575	Insoluble
16	3.985	Insoluble

[a] Aqueous solubility was measured by adding each analogue to water at a final theoretical concentration of 100 μM. The samples were then clarified by centrifugation, and the absorbance (at 350 nm, determined in DMSO-solubilized aliquots prepared in parallel) was measured.

stimulatory activity. Other modifications on the anthranilate ring (Fig. 6, compounds 12 to 16) did not substantially enhance the MV-stimulatory activity of the molecules.

Previous works have shown that most of the PQS made by *P. aeruginosa* is packaged into MVs (13, 17), raising the possibility that specific structural features of the molecule might affect interaction with the cell envelope by affecting its lipophilicity. A chemical informatics approach (www.molinspiration.com/cgi-bin/properties) was therefore used to calculate the octanol-

water partition coefficient (logP value) for each analogue. In parallel, we also measured the solubility of each analogue in water. There was a good correlation between these parameters (Table 1). Notably, the least active compounds (1, 7, and 8) showed the greatest water solubility, consistent with there being a minimum lipophilicity required for PQS. However, lipophilicity alone does not account for the dramatic functional impact of subtle structural variations. For example, extension of the alkyl chain from 8 to 9 carbon atoms (Table 1, cf. analogues 4 and 5) increases the logP value but abolishes pyoverdine stimulation.

Overall, the correlation between MvfR binding and pyoverdine stimulation was moderate. When the induction ratios for each assay are plotted against one another, two distinct clusters are observed (Fig. 7). Compounds 4, 6, 9, 10, 11, 14, and PQS all showed good inducing activity in both assays (cluster I),
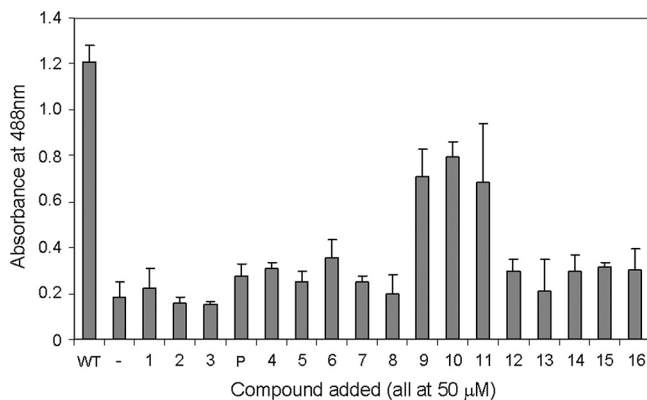

FIG. 6. Membrane vesicle formation in the presence of PQS analogues. Cultures of PA14 or the isogenic *pqsB* mutant (DH125) were grown with good aeration (200 rpm in 250-ml flasks) at 37°C in 20 ml brain heart infusion (BHI) medium. The DH125 cultures contained DMSO alone (−) or 50 μM indicated analogues. After 18 h, the cultures were harvested and clarified by sedimentation at 5,000 × *g* (20°C) for 10 min, and the clarified supernatants were passed through a 0.2-μm filter. Aliquots (1-ml volume) of the filtered supernatant were sedimented for 2.5 h (4°C) at 800,000 × *g* in a Beckman Optima benchtop ultracentrifuge (MLA-130 rotor). The supernatant was removed by aspiration, and the pellet was washed once in 50 mM NaCl. The pellets were air dried and resuspended in 30 μl water. The phospholipid content of each pellet was measured using the ammonium ferrothiocyanate method of Stewart (20). The data represent the averages from the results of 2 independent biological repeats. WT, wild type (the MV content of PA14); P, PQS.
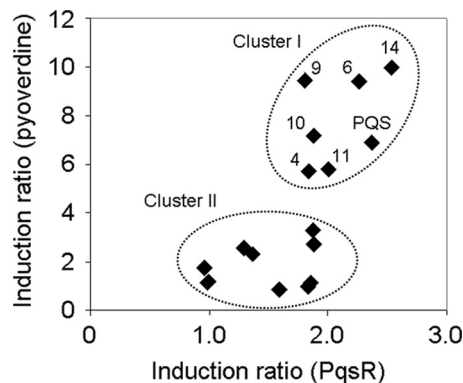

FIG. 7. Quantitative comparison of PQS analogue activity in MvfR-dependent and MvfR-independent assays. The induction ratios (activity in the presence of the analogue/activity in the absence of the analogue) were calculated using the data shown in Fig. 2 and 3 and then plotted against one another. The identities of the active analogues in cluster I are indicated.

indicating that these modifications to PQS are in structurally tolerant positions. Interestingly, some members of cluster I were also better at stimulating MV production than the compounds in cluster II. The latter were less active, indicating that if (i) the alkyl chain is too short (analogues 1 to 3) or too long (analogue 5), (ii) the alkyl group is substituted by a phenyl moiety (analogues 7 and 8), (iii) the anthranilate ring carries electron-donating groups (analogues 13, 15, and 16), or (iv) position 8 in the anthranilate ring carries an electronegative atom (analogue 12), the specific signaling or MV-promoting activity of the molecule becomes compromised. Therefore, almost every part of the PQS molecule contributes in some way toward its function as a signaling molecule. Alternatively, these specific structural factors may diminish the bioavailability of the molecules. We also note that, with the exception of the water-soluble analogues (1, 7, and 8), all members of cluster II retained PQS-synergistic activity, indicating that this function of the molecule is more dependent on its general properties (e.g., lipophilicity) rather than specific chemical groups.

Our results further support the notion that the structure of PQS is constrained through interaction with multiple factors (currently known to include LPS, MvfR, and iron). Furthermore, it is possible that additional PQS receptors remain to be identified. In this regard, both *Burkholderia thailandensis* and *Burkholderia pseudomallei* contain functional *pqsABCDE* homologues (7, 21), but neither of their genomes encodes a convincing MvfR homologue.

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