The Effect of a Cell-to-Cell Communication Molecule, Pseudomonas Quinolone Signal (PQS), Produced by P. aeruginosa on Other Bacterial Species

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One of the most important factors in the development of a bacterial community is whether the bacteria are able to grow in that habitat. The regulation of bacterial growth is generally studied in relation to physicochemical conditions, however, how bacterial communities regulate themselves remains unclear. In our previous study, it was demonstrated that a cell-to-cell communication molecule, 2-heptyl-3-hydroxy-4-quinolone, referred to as the Pseudomonas quinolone signal (PQS), affects respiring-activity in Pseudomonas aeruginosa without requiring its cognate receptor PqsR. The results suggested that PQS may affect other bacterial species, which was further examined in this study. PQS repressed the growth of several species including both Gram-negative and Gram-positive bacteria. In most cases, this effect differed from the bacteriostatic or bacteriolytic actions of antibiotics. The growth repression by PQS was inhibited when iron was added to the medium, indicating iron-chelating activity to be involved. In addition, PQS affected oxygen consumption in some species tested, and may have other underlying effects. Thus, this cell-to-cell communication molecule may influence the development of bacterial communities by regulating bacterial growth, and physicochemical factors such as iron would be important in determining its effect.

Key words: Pseudomonas quinolone signal, cell-to-cell communication, interspecies interaction

Studies of natural samples using techniques such as PCR-denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism have provided information about the kinds of bacteria inhabiting different environments. However, why bacteria live where they do is still unclear. The regulation of growth is important when considering the habitats of bacteria, and many factors that regulate energy production have been studied, such as the presence of electron acceptors or electron donors and pH.

Besides physicochemical factors, extracellular bacterial metabolites are also reported to control the growth of bacteria. For example, indole derivatives were isolated as growth inhibitors in Symbios bacterium thermophilum (35). Antibiotic reactions are another example of an interaction where bacterial growth is inhibited by extracellular bacterial metabolites. Recently, it has been proposed that antibiotics work as signals, regulating gene expression (8). Moreover, numerous bacteria utilize cell-to-cell communication signaling molecules, enabling them to coordinate diverse biological responses (27). In Gram-negative bacteria, these signaling molecules are typically N-acyl-L-homoserine lactones (AHLs). Pseudomonas aeruginosa is a well-studied bacterium possessing at least two AHL-dependent quorum-sensing systems, the LasR-LasI (las) and the RhlR-RhlI (rhl) systems (24). In addition, a non-AHL quorum-sensing signal, 2-heptyl-3-hydroxy-4-quinolone, referred to as the Pseudomonas quinolone signal (PQS), was found in P. aeruginosa (25). PQS regulates the production of virulence factors such as pyocyanin (14), and also regulates production of 2-alkyl-4-quinolones (AHQs), such as 2-heptyl-4-quinolone N-oxide (HQNO) and the precursor of PQS, 2-heptyl-4-quinolone (HQQ), as a result of the transcription of the pqsA-E operon (12). The regulation of the pqsA-E operon is mediated by its cognate receptor PqsR (MvfR) (33). In addition, PQS has several unique features, one of which is its iron-chelating activity. Rapid loss of free iron was observed when PQS was added to medium, and it was determined that PQS chelates Fe (III) in a 3:1 complex (3, 11). Moreover, PQS has been demonstrated to possess both anti-oxidant and pro-oxidant activity, though it has been proposed to primarily be an anti-oxidant, with the presence of Fe (III)/Fe (II) inducing a pro-oxidant effect (16). PQS has also been demonstrated to associate with cells (18), and to interact with lipopolysaccharide and the phosphate lipid component of the outer membrane (21).

Previously, we demonstrated that these cell-to-cell communication signals affect denitrifying activity in P. aeruginosa (31, 32). The AHL signals regulate denitrification (34, 37), by influencing the denitrifying genes through their cognate receptors, and the las quorum-sensing system regulates denitrification dependent on the rhl quorum-sensing system (31). PQS however does not regulate denitrifying genes but affects the activities of denitrifying enzymes and the NO3−-respiratory chain, without requiring its receptor PqsR (32). The mechanism by which it inhibits denitrification in P. aeruginosa suggests that PQS may affect respiratory activity in species other than P. aeruginosa. In this study, we examined comprehensively the effect of PQS on other bacterial species, especially its influence on growth, in order to gain insights into the role of bacterial interactions in the control of bacterial populations and development of bacterial communities.

Our results indicate that PQS affects the growth of both

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Gram-negative and Gram-positive bacteria, with its iron-chelating activity playing an important role. The effect on growth was not like that of antibiotics which act in a bacteriostatic or bacteriolytic fashion, but instead caused the bacteria to grow at a slower rate. We suggest that this manner of controlling bacterial populations may become significant in controlling the development of bacterial communities.

Materials and Methods

Bacterial strains and growth conditions

P. aeruginosa PAO1, Pseudomonas putida AC10 and Escherichia coli K12 were grown at 37°C, and Pseudomonas stutzeri Zobell, Comamonas terrigena NBRC 13111, Deftia acidovorans NBRC 14903 were grown at 30°C in Luria-Bertani (LB) medium or on LB agar plates. Mutants of P. aeruginosa PAO1 and plasmids were constructed in another study (32). Soil samples were collected from University of Tsukuba. Strains isolated from acidovorans

\[ \text{MgSO}_4 \cdot 7\text{H}_2\text{O}, 0.3\% \text{ (w/v) casamino acids}, 10 \text{ mg of CaCl}_2 \cdot 2\text{H}_2\text{O}, 5 \text{ mg of MnSO}_4 \cdot 4\text{H}_2\text{O}, 2 \text{ mg of CuSO}_4 \cdot 7\text{H}_2\text{O} \text{ and 2 mg of ZnSO}_4 \cdot 7\text{H}_2\text{O per liter (29). Cells were incubated in this medium until they entered a stationary phase and the supernatant of the culture was then collected. The supernatant was adjusted to pH 6.0 and analyzed with the Chrome azurol S (CAS) assay (28).}

Sequence analysis

The bacterium-specific and universal primers 27F(5'-AGAGTTT GATCCTGCGTCAAG-3’) and 1494R(5'- TACGGAGTCAGGTGA C CTTGTTA-3’) (where Y is C or T) were used to amplify the 16S rRNA gene region between nucleotides 52 and 1479 (E. coli numbering) (22). Fifty nanograms of PCR product was used for sequencing with the Bigdye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) and the 27F primer. The products were sequenced on a 3130 Genetic Analyzer (Applied Biosystems). Approximately 600 bp of the 16S rRNA gene sequence was compared to known sequences in the DNA Data Bank of Japan (DDBJ).

Nucleotide sequence accession numbers

The nucleotide sequence data reported here are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB306465 to AB506476.

Results

PQS affects growth in species other than Pseudomonas aeruginosa

In P. aeruginosa, PQS extended the lag phase and decreased growth rates under aerobic conditions (9, 16, 32). Moreover, it affected denitrification without requiring its cognate receptor, PqsR (32). These results prompted us to examine the effect of PQS in other species. PQS was added to the medium of isolated bacteria thought to share the same natural habitat as P. aeruginosa, and its effect on growth was measured. The strains were grown aerobically in 4-mL LB medium using 20-mL test tubes. Growth was repressed in all the strains tested, though the extent of the repression varied (Fig. 1). The growth of C. terrigena NBRC 13111 was particularly affected (Fig. 1B), whereas that of E. coli K12 was repressed only slightly. The effect on growth in P. putida AC10 was observed not only under aerobic conditions, but also in anaerobic cultures in which NO\(_3^−\) was used as the electron acceptor. Under anaerobic conditions, PQS repressed the growth of P. putida AC10, yielding a 10-fold lower final OD\(_{600}\) in the stationary phase.

Addition of iron prevents the effect of PQS on growth

In a recent study, PQS had a pro-oxidant effect resulting in the lysis of P. aeruginosa cells (16). To test whether the growth repression was due to the production of reactive oxygen species, an anti-oxidant (5 mM L-proline) was added in the medium in addition to PQS. However, the growth rate did not recover in any of the strains tested (data not shown).
Furthermore, there were no significant differences in the number of CFUs between agar plates with PQS added and agar plates without PQS added when stationary phase cultures were plated, which suggests that PQS did not induce the lysis of these cells (data not shown).

PQS has been reported to chelate iron (3, 11). Therefore, we next tested the effect of iron on the growth repression. When FeCl\(_3\) was added to medium containing PQS, growth was restored in all bacteria tested (Fig. 2). The same results were obtained for \(P. \) putida AC 10, under anaerobic conditions (data not shown). Thus, iron appears to play an important role in the growth repression, and the effect may be due to the iron-chelating activity of PQS.

**Effect of PQS on oxygen consumption**

In our recent study, it was demonstrated that PQS inhibits NO\(_3^-\)-respiration in \(P. \) aeruginosa (32). Likewise, PQS may repress respiratory activity of other bacterial species. To examine this, the oxygen consumption rate was measured. Strains cultured in LB with or without 50 \(\mu\)M PQS were collected during the early stationary phase and the oxygen consumption rate was measured. FeCl\(_3\) was added to the medium at a final concentration of 50 \(\mu\)M. The plasmid pUCP24 was transferred into each \(P. \) aeruginosa PAO1 strain as a control for the complementation experiments. Data represent the mean±SD for three independent experiments.

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**Fig. 1.** Effect of PQS on growth of bacteria other than \(P. \) aeruginosa. Aerobic growth of (A) \(P. \) stutzeri ZoBell, (B) \(C. \) terrigena NBRC 1311\(^T\), (C) \(D. \) acidovorans NBRC 1490\(^T\), (D) \(E. \) coli K12 and (E) \(P. \) putida AC10 in LB medium. PQS was added to the medium at a final concentration of 50 \(\mu\)M. Data represent the mean±SD from three independent experiments.

**Fig. 2.** Effect of iron on the growth inhibition. Strains were inoculated at an OD\(_{600}\) of 0.01 and growth was measured over a 12-h period. PQS and FeCl\(_3\) were each added to the medium at a final concentration of 50 \(\mu\)M. Data represent the mean±SD for three independent experiments.

**Fig. 3.** Effect of PQS on oxygen consumption rates in several bacterial species. Effect of PQS on the oxygen consumption rate of (A) \(P. \) stutzeri ZoBell, (B) \(D. \) acidovorans NBRC 1490\(^T\), (C) \(E. \) coli K12, (D) \(P. \) putida AC10 and (E) \(P. \) aeruginosa PAO1. Strains cultured in LB with or without 50 \(\mu\)M PQS were collected during the early stationary phase and the oxygen consumption rate was measured. FeCl\(_3\) was added to the medium at a final concentration of 50 \(\mu\)M. The plasmid pUCP24 was transferred into each \(P. \) aeruginosa PAO1 strain as a control for the complementation experiments. Data represent the mean±SD for three independent experiments.

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Furthermore, there were no significant differences in the number of CFUs between agar plates with PQS added and agar plates without PQS added when stationary phase cultures were plated, which suggests that PQS did not induce the lysis of these cells (data not shown).
added to the culture of \textit{P. stutzeri} ZoBell or \textit{D. acidovorans} NBRC 1490\textsuperscript{T} (Fig. 3A and B), while the growth of these strains was repressed by PQS (Fig. 1A and C). These results suggest that there are other underlying mechanisms in the effect of PQS on growth. \textit{C. terrigena} NBRC 1311\textsuperscript{T} was not tested due to its poor growth in the presence of PQS.

\textit{Regulation of oxygen consumption by PQS in Pseudomonas aeruginosa}

Oxygen consumption was also tested in \textit{P. aeruginosa} PAO1. In \textit{P. aeruginosa} PAO1, the oxygen consumption rate was higher in a $\Delta pqsA \Delta pqsR$ deletion strain than in the wild-type (WT) strain (Fig. 3E). Similar results were observed using a $\Delta pqsH$ mutant, which produces AHQs related to PQS but does not produce PQS (data not shown). When PQS was added to the $\Delta pqsA \Delta pqsR$ strain, oxygen consumption rate was not repressed to the level of PAO1 WT strain, suggesting that the PQS receptor, PqsR, is required for the regulation of oxygen consumption. Indeed, the oxygen consumption rate was reduced when a plasmid carrying $pqsR$ was transferred into the cell (Fig. 3E).

\textit{Effect of PQS on soil samples}

Since PQS repressed growth in several species tested, we attempted to examine its effect more broadly using soil samples. Soil collected from the grounds of University of Tsukuba was suspended in saline and plated on 1/10$\times$NB agar medium with or without PQS, and colonies were counted. CFUs were measured for 5 d after the soil suspension was plated. On the first day, the number of CFUs was ten times less on the plates with PQS than on those without PQS (Fig. 4A). The number was restored when FeCl\textsubscript{3} was added in addition to PQS, further supporting our finding that the presence of iron inhibits the effect of PQS on bacterial growth.

After 2 d, the number of CFUs on the agar plates with PQS reached that in the control medium. To examine whether the composition of bacterial colonies growing on the PQS-containing plates was identical to that of colonies growing in the control medium, all of the colonies from plates incubated 5 d were removed with a toothpick and analyzed by PCR-DGGE. The V3 region from the 16S rRNA gene was amplified by PCR and separated by gradient gel electrophoresis. Three independent experiments were carried out using the same soil sample. Although specific bands appeared in some of the samples, reproducibility was low among the three experiments. Taking into account this difference among samples plated under the same conditions, PQS did not significantly alter the composition of the bacteria culturable on 1/10$\times$NB agar plates (Fig. 4B). Likewise, the banding pattern of samples grown with PQS and FeCl\textsubscript{3} did not differ significantly from that of samples grown on the PQS-added plate or the control plate. These results suggest that the decrease in CFUs caused by PQS was simply due to growth repression of the bacteria that can grow on the 1/10$\times$NB agar and iron recovered the growth of those bacteria.

\textit{Isolation of PQS-susceptible and non-susceptible strains}

To obtain more information about the range of strains susceptible to PQS, colonies from the agar plate where the soil suspension was plated were isolated. Five colonies that grew within 24 h (PQS-1, PQS-2, PQS-3, PQS-4, and PQS-5) and 7 colonies that grew after 24 h (PQS-7, PQS-8, PQS-9, PQS-10, PQS-11, PQS-12, and PQS-13) were removed and tested against PQS again. The strains were cultured clonally in liquid medium, then diluted to approximately $10^4$ cells mL$^{-1}$ and 2 $\mu$L of each dilution was spotted on agar plates with or without PQS. PQS-1, PQS-2, PQS-4 and PQS-5 formed colonies within 24 h in the PQS-containing plates whereas PQS-7, PQS-8, PQS-10, PQS-11, PQS-12 and PQS-13 did not. The ability of PQS-3 and PQS-9 to form colonies varied among the experiments (Table 1). In all cases, colonies were observed within 24 h when FeCl\textsubscript{3} was added to the medium with PQS (data not shown). Similar results were obtained.
Inter-Species Effect of PQS on Growth

when the effect of PQS was tested in liquid cultures (Fig. 5). As in the other experiments, the effect of PQS was inhibited by FeCl₃. The isolated strains were identified by sequencing their 16S rRNA gene and the results are shown in Table 1. These results demonstrate that PQS’s effect covers a broad spectrum of species, ranging from Gram-negative to Gram-positive bacteria.

### Table 1. Effect of PQS on growth of soil isolates

| Strains     | Relative species (% similarity) | Growth inhibition by PQS
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>PQS-1</td>
<td>Klebsiella sp. (100)</td>
<td>−</td>
</tr>
<tr>
<td>PQS-2</td>
<td>Arthrobacter sp. (100)</td>
<td>−</td>
</tr>
<tr>
<td>PQS-3</td>
<td>Rhodococcus erythropolis (99)</td>
<td>±</td>
</tr>
<tr>
<td>PQS-4</td>
<td>Enterobacter sp. (99)</td>
<td>−</td>
</tr>
<tr>
<td>PQS-5</td>
<td>Erwinia persicina (98)</td>
<td>−</td>
</tr>
<tr>
<td>PQS-7</td>
<td>Comamonas sp. (97)</td>
<td>+</td>
</tr>
<tr>
<td>PQS-8</td>
<td>Bacillus cereus (99)</td>
<td>+</td>
</tr>
<tr>
<td>PQS-9</td>
<td>Pseudomonas sp. (99)</td>
<td>±</td>
</tr>
<tr>
<td>PQS-10</td>
<td>Rhodococcus globulatus (100)</td>
<td>+</td>
</tr>
<tr>
<td>PQS-11</td>
<td>Pseudomonas sp. (99)</td>
<td>+</td>
</tr>
<tr>
<td>PQS-12</td>
<td>Stenotrophomonas sp. (100)</td>
<td>+</td>
</tr>
<tr>
<td>PQS-13</td>
<td>Pseudomonas viridiflava (100)</td>
<td>+</td>
</tr>
</tbody>
</table>

*Results of three independent experiment are demonstrated. (−) growth was not inhibited by PQS, (+) growth was inhibited by PQS, (±) results varied among the three experiments.*

**Discussion**

Bacteria face not only physicochemical signals but also the signals of surrounding bacterial species. To understand the habitat of bacteria, it is important to study such interactions. PQS functions as a cell-to-cell communication signal that regulates virulence factors in *P. aeruginosa* (14). In addition, it is suggested to act physically in membrane vesicle production (20, 21), and has been demonstrated to chelate iron (3, 11). Moreover, recent studies have demonstrated bioactivity of PQS against human cells (5, 15, 30). The aim of
this study was to analyze the effect of PQS on bacterial species other than *P. aeruginosa*, with an emphasis on its effect on growth. We found that PQS influences the growth of several species, including Gram-negative and Gram-positive bacteria, suggesting it has a broad impact in controlling bacterial communities. Thus, in addition to the virulence factors it regulates, PQS itself affects the growth of other species, indicating its potential function as a cell-to-cell communication molecule.

A cell-to-cell communication molecule produced by *P. aeruginosa*, N-(3-oxododecanoyl)-l-homoserine lactone, was reported to have bactericidal activity against Gram-positive bacteria (17). Recently, it was suggested that antibiotics act as signaling molecules at sub-inhibitory concentrations (13). Here we demonstrate that another cell-to-cell communication molecule produced by *P. aeruginosa* affects the growth of other bacterial species. This finding may help us to better understand the relationship between antibiotics and cell-to-cell signaling molecules. Of note, PQS’s effect on growth was different from that of antibiotics which act bacteriostatically or bacteriolytically, in that it had a tendency to slow the growth rate. This was also confirmed by experiments using soil samples in which little change in bacterial composition was observed when PQS was added to the medium. PQS’s inhibitory effect on bacterial growth may be relevant in controlling the development of a bacterial community structure.

A PQS-related AHQ, HQNO, has been reported to inhibit growth in Gram-positive bacteria (19). Compared to HQNO, PQS has a broad spectrum of targets, affecting both Gram-negative and Gram-positive bacteria. It would be interesting to examine further whether the production of PQS and its related AHQs are regulated in response to these bacterial species. In fact, HHQ, another precursor of PQS, has been reported to be produced in Gram-negative bacteria such as *Burkholderia pseudomallei*, *Burkholderia thailandensis*, *Burkholderia cenocepacia* and *P. putida* (10), which suggests that the presence of these bacteria could induce PQS production in *P. aeruginosa*, since HHQ is taken up and further converted into PQS (12). The induction of PQS production in the presence of other bacteria may give an advantage to *P. aeruginosa* and allow it to dominate the environment.

PQS’s growth repression of other species was inhibited by the addition of iron, indicating the importance of its iron-chelating activity. Considering the effect of iron, if PQS depleted iron from the medium, the addition of iron would restore levels. Moreover, iron has been reported to reduce the clinical effect of several drugs, such as tetracycline, through the formation of iron-drug complexes (1). Thus, iron may inhibit the effect of PQS on cells by forming an iron-PQS precipitate. At this point, it is hard to know whether adding iron restored depleted levels in the medium or reversed the PQS-mediated growth inhibition due to the precipitation of an iron-PQS complex. Still, it is significant that the concentration of iron in the environment determines the effect of PQS on other bacteria.

Since iron was a key factor in the effect of PQS and a previous study demonstrated that the growth of a *P. aeruginosa* mutant which does not produce siderophore was greatly repressed compared to the WT strain (11), we hypothesized that siderophore production determines susceptibility to PQS. However, no correlation was observed between the strains in which growth was repressed by PQS and those in which siderophore was produced. Therefore, other factors could be involved in susceptibility to PQS. Quinolone antibiotics, related in structure to PQS, are effluxed out of cells by pumps in certain bacteria (26). Therefore, efflux pumps may play a role in susceptibility to PQS. Another possibility is that PQS is degraded in some strains; however, this could not be observed by detecting PQS with thin layer chromatography (data not shown).

In addition to PQS-mediated growth repression, another effect observed in *E. coli* K12 and *P. putida* AC10 was a repression of oxygen consumption. Interestingly, there was no clear correlation between the growth repression (Fig. 1) and the reduction in the oxygen consumption rate (Fig. 3), as oxygen consumption was not affected by PQS in *D. acidovorans* NBRC 1490 and *P. stutzeri* ZoBell, whose growth was repressed by PQS. The mechanisms by which PQS represses growth and oxygen consumption may be different. These results, along with reports of bio-activity of PQS against human cells (5, 15, 30) lead us to suspect that PQS has some other underlying bio-activity against bacterial cells.

In our previous study, PQS inhibited nitrate respiration, and this effect did not require the regulator PqsR (32). In contrast, the effect of PQS on oxygen consumption required PqsR. Thus, these results suggest that PQS affect aerobic and anaerobic respiration in *P. aeruginosa* PAO1 differently. Therefore, the target of PQS in the respiratory chain may not be a component such as NADH-dehydrogenase that is common to both aerobic and anaerobic respiration (6). Alternatively, PQS may induce production of some factors under aerobic conditions that inhibit the effect of PQS observed under anaerobic conditions.

While we focused on the effect of PQS on growth, it would also be interesting to know whether PQS acts as a signal in bacterial species other than *P. aeruginosa*. The PQS receptor, PqsR, interacts with molecules other than PQS, such as HHQ and farnesole (7, 36), which further suggests there to be receptors other than PqsR that interact with PQS.

Several studies have reported that signaling molecules such as cyclic AMP or AHLs enhanced the culturability of natural samples (4). As shown by the PCR-DGGE banding pattern (Fig. 4B), no such effect was observed in our study; however, further experiments using various samples will be required to conclusively determine the effect of PQS on bacterial culturability.

In summary, our study demonstrates that a previously identified cell-to-cell communication molecule, PQS, affects the growth of bacterial species other than *P. aeruginosa*. The mechanism by which growth is affected seems to involve multiple pathways and may vary among species. One environmental key factor to this interaction is iron, which suggest that bacterial interactions are controlled by the surrounding environment. Elucidating these interactions controlling bacterial growth may lead to a better understanding of the development of bacterial communities, and further supply basic information for improving the culturability of bacterial cells from natural samples.
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