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A stable isotope dilution assay for the quantification of the *Pseudomonas* quinolone signal in *Pseudomonas aeruginosa* cultures

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Abstract

A stable isotope dilution method was developed to analyse 2-heptyl-3,4-dihydroxyquinoline, also called the *Pseudomonas* quinolone signal (PQS), directly in *Pseudomonas aeruginosa* cultures by liquid chromatography coupled to mass spectrometry (LC/MS). PQS, along with the isobaric 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), were quantified in various *Pseudomonas* liquid cultures using a deuterated PQS analog as internal standard. The kinetic of production of these quinolines in a growing culture of *P. aeruginosa* PA14 showed that their production starts at the end of the logarithmic growth phase and is maximal at the onset of the stationary growth phase. The concentration of PQS reached a maximum at 13 mg/l and then decreased, while the HQNO concentration reached 18 mg/l and then remained stable. Culture supernatants of *P. aeruginosa* strains PAO1 and PA14 produced similar concentrations of PQS whereas no PQS or HQNO could be detected in culture supernatants of the *P. aeruginosa* strain PAK or in the other *Pseudomonas* species tested, including phytopathogenic pseudomonads.

Keywords: PQS; Stable isotope; Mass spectrometry; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa, a ubiquitous Gram-negative bacterium, is an important opportunistic pathogen primarily infecting immunocompromised hosts and those with severe burns [1]. It is also the main infectious agent and a major cause of mortality for cystic fibrosis patients [2]. *P. aeruginosa* produces numerous extracellular virulence factors, such as proteases, toxins, and lipases. The synthesis of a large number of these exoproducts is controlled in a cell density-dependent manner using small diffusible intercellular signaling molecules (autoinducers), a process known as "quorum sensing" (QS) [3]. Two separate QS systems (termed *las* and *rhl*), each of which consisting of a transcriptional regulator (LasR or RhlR) and a cognate autoinducer synthase (LasI or RhlI) producing a specific *N*-acyl-L-homoserine lactone (AHL), modulate gene transcription in response to increasing AHL concentrations [4]. Together, these two systems comprise a hierarchical cascade (where the *las* system influences the *rhl* system) that coordinates the expression of numerous genes, as well as the production of additional QS signals [5]. A third intercellular signaling molecule, the *Pseudomonas* quinolone signal (PQS), identified as 2-heptyl-3,4-dihydroxyquinoline, may not be directly implicated in sensing population density per se but is nevertheless involved in the QS hierarchy [6]. PQS was suggested to act by up-regulating the *rhl* system [7]. However, recent observations indicate that PQS signaling might directly control the expression of genes regulated by the *rhl* QS system [8]. PQS production requires both an active *lasR* [7] and the recently reported transcriptional regulator MvfR [9].

PQS has been detected in the lungs of chronically infected cystic fibrosis patients with a qualitative approach using thin-layer chromatography [10]. Until now, two methods of PQS quantification have been published. One is an indirect biological assay, which monitors the expression of a *lasB'-lacZ* fusion upon exposure to PQS [6], and the second is a thin-layer chromatography separation method followed by densitometric analysis [11,12]. Because of the inherent limitations and uncertainties involved with both assays and the difficulty of automating the second method, a

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fast and reliable assay was required. We developed a stable isotope dilution method to quantify PQS by liquid chromatography coupled to mass spectrometry (LC/MS). This allowed us to determine that PQS is maximally produced during the early stationary phase of growth and that a significant proportion of this PQS is apparently binding to the surface of the bacterial cells.

2. Material and methods

2.1. Microorganisms

Pseudomonas aeruginosa strains PA14, PAO1 (from B.H. Iglewski, Univ. of Rochester, Rochester, NY), and PAK (from J.S. Mattick, Univ. of Queensland, Brisbane, Australia), *P. fluorescens* strain 2-79, *P. syringae* pv. maculicola strain ES4326 and pv. tomato strain DC3000 (both from F.M. Ausubel, Massachusetts General Hospital, Boston, MA) and *P. fragi* strain ATCC 27363 were investigated.

2.2. Cultivation and samples preparation

The kinetics of production of PQS by strain PA14 was performed in triplicate in 250-ml Erlenmeyer flasks containing 50-ml cultures in LB medium. Flasks were incubated at 37 °C in an orbital shaker at 240 rpm. Cultures were inoculated with 2 ml of a 12-h culture in LB in order to obtain a starting $OD_{600} = 0.2$. A 2-ml aliquot was taken at regular time intervals and growth was measured by the OD at 600 nm. For PQS analysis, each sample was diluted with an equivalent volume of methanol containing the internal standard (20 mg/l of 5,6,7,8-tetradeutero-2-heptyl-3,4-dihydroxy-quinoline) and 2% acetic acid. The solution was then centrifuged at $13{,}000 \times g$ for 15 min and the supernatant transferred to a borosilicate vial kept at 4 °C.

The production of PQS by various *P. aeruginosa* strains and other *Pseudomonas* species was determined from 6-ml Luria-Bertani (LB) broth cultures in 20 × 150-mm test tubes incubated at 37 °C (*P. aeruginosa*) or 30 °C (other strains) for 16 h in a rollerdrum. The cell-free culture supernatant was obtained by centrifugation then extracted twice with the same volume of ethyl acetate, the solvent evaporated, and the residue dissolved in methanol containing the internal standard.

2.3. Synthesis of labeled PQS

The labeled PQS used as internal standard, the 5,6,7,8-tetradeutero-2-heptyl-3,4-dihydroxyquinoline, was synthesized as described in Fig. 1. Briefly, methyl 3-ketodecanoic ester was obtained from the addition of octanoyl chloride unto 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) and treatment of the resulting mixture with refluxing methanol, as described by Oikawa et al. [13]. The keto ester was reacted with pentadeuteroaniline to produce an intermediate enamine [14], which was then cyclised in sulfuric acid and acetic anhydride [15]. The substituted quinoline obtained was formylated at the 3 position with hexamethylenetetramine and the formyl group replaced by an hydroxyl group by oxidation with hydrogen peroxide in basic medium, as described by Pesci et al. [6].

2.4. Analytical conditions

Analyses were performed with an Agilent HP1100 (Agilent Can., Pointe-Claire, Canada) coupled to a Micromass Quattro II (Micromass Canada, Pointe-Claire). The column was a 4.6 × 150-mm Agilent HP Eclipse XDB-C8 using a water/acetonitrile gradient with a constant 1% concentration of acetic acid. The initial solvent composition was 80% water:20% acetonitrile, which was linearly changed to 2% water:98% acetonitrile in 28 min, kept stable for an additional 6 min, returned to the initial values in 1 min, and finally left to stabilize for another 3 min. The flow rate was 0.4 ml/min, which was split to 40 μl through a Valco Tee splitter. The volume of the sample injected was 20 μl. The analyses were performed in positive electrospray ionization mode. The capillary voltage was 3 kV and the cone voltage, 21 V. The source temperature was 120 °C. Nitrogen was

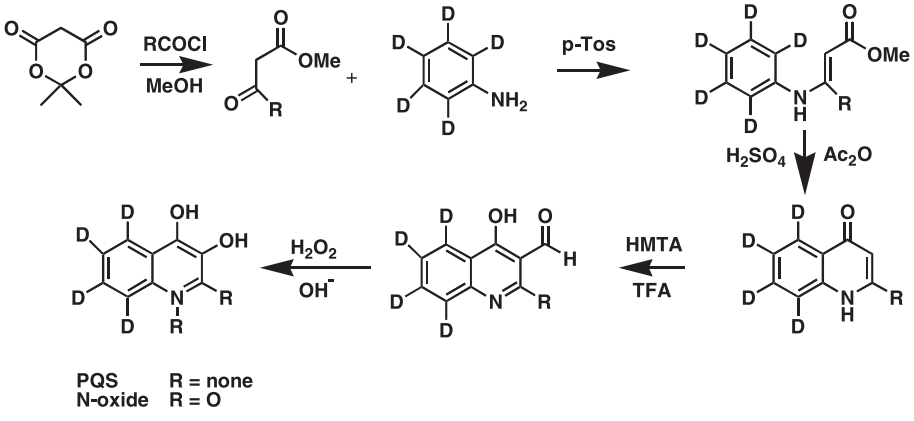

Fig. 1. Schematic of the synthetic pathway for labeled PQS.

used as a nebulising and drying gas at flow rates of 20 and 200 ml/min, respectively. Data were collected in Selective Ion Detection (SID) mode using the *m*/*z* 260 and 264 ions. Quantification of PQS in PA14 cultures was performed by measuring the relative area of the chromatograms of the pseudomolecular ions of PQS and of the internal standard and multiplying the ratio by the concentration of the internal standard added. Measurements of PQS in PAO1 and the other pseudomonads tested along with their PA14 controls were performed in full scan mode and quantitated relative to the internal standard.

3. Results

In order to develop a reliable assay for measuring PQS concentrations in liquid bacterial cultures, a deuterated PQS analog was needed to be used as an internal standard. A protocol was designed allowing straightforward synthesis in quantities of the order of 350 mg per round of synthesis. The keto ester was made by acylating Meldrum's acid followed by methanolysis instead of using an ester of acetoacetic acid, as initially described by Wells [16], because of the excellent yields obtained by Oikawa et al. [13]. Cyclisation of the intermediate enamine was also performed with sulfuric acid in acetic anhydride rather than by simple thermal cyclisation in diphenyl ether, as initially described by Leonard et al. [17]. Higher yields and ease of purification were obtained in our hands using the former method.

LC/MS analysis showed that the retention time at the apex of the chromatographic peak of the deuterated PQS was consistently smaller by 4 s compared to PQS, as is often observed for deuterium-labeled compounds. The mass spectra of the two compounds are presented in Fig. 2. The molecular weights of PQS and deuterated PQS are 259 and 263, respectively, and, under these analytical conditions, only the $[M+H]^{+1}$ pseudomolecular ions were observed at *m*/*z* 260 and 264. Calibration curves for deuterated PQS and HQNO (see below) were obtained for concentrations up to 20 mg/l and found to be linear with correlation coefficients of 0.993 and 0.994, respectively. The limit of quantification was 0.12 mg/l for a 1:3 signal-to-noise ratio. The response factor of HQNO was 0.897 that of PQS.

Initial analysis of PA14 culture supernatants in full scan mode revealed a large number of chromatographic peaks (Fig. 3). In SID scanning mode, an additional chromatographic peak was observed at a shorter retention time, which presented the same *m*/*z* 260 ion than PQS. MS/MS analysis of this other compound, isobaric with PQS, showed that it was 2-heptyl-4-hydroxyquinoline N-oxide (HQNO, R=O, Fig. 1), and this was confirmed using an authentic standard (Sigma-Aldrich, Oakville, ON, Canada). Because the MS/MS spectrum of HQNO presented the same ions as the structurally very similar PQS, although with different relative intensities, no further attempts were made to use an MS/MS direct infusion method to quantitate PQS. The use of a relatively slow gradient allowed a 2-min separation between PQS and HQNO, which provided baseline separation allowing proper quantification of both compounds (Fig. 3b).

We determined that PQS has a maximum solubility of only 1 mg/l in water at pH 7, and of 5 mg/l in LB. Being very hydrophobic, PQS is likely to have a high affinity for the lipid-rich membrane of bacterial cells. To overcome both problems without resorting to an extraction, an equal volume of methanol was added to the culture sample prior to centrifugation. This procedure was tested with 20%, 50%, 75% and 88% final methanol concentrations. At 20% methanol concentration, only 20% of added PQS could be recovered and at concentrations above 50% the concentration of PQS observed remained reproducible with a recovery of 94% of the added labeled PQS. To confirm the attachment of PQS to cells, three *P. aeruginosa* cultures

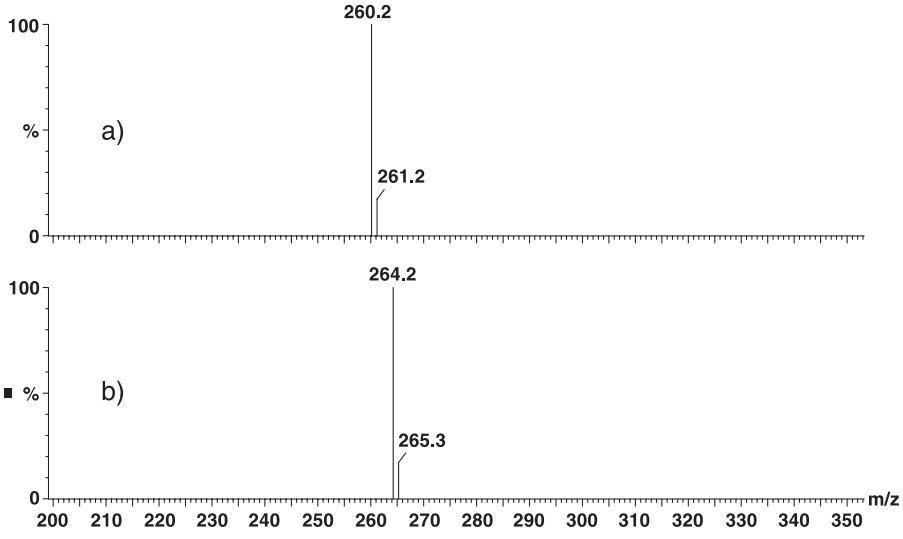

Fig. 2. Positive electrospray mass spectra of (a) PQS and (b) labeled PQS.

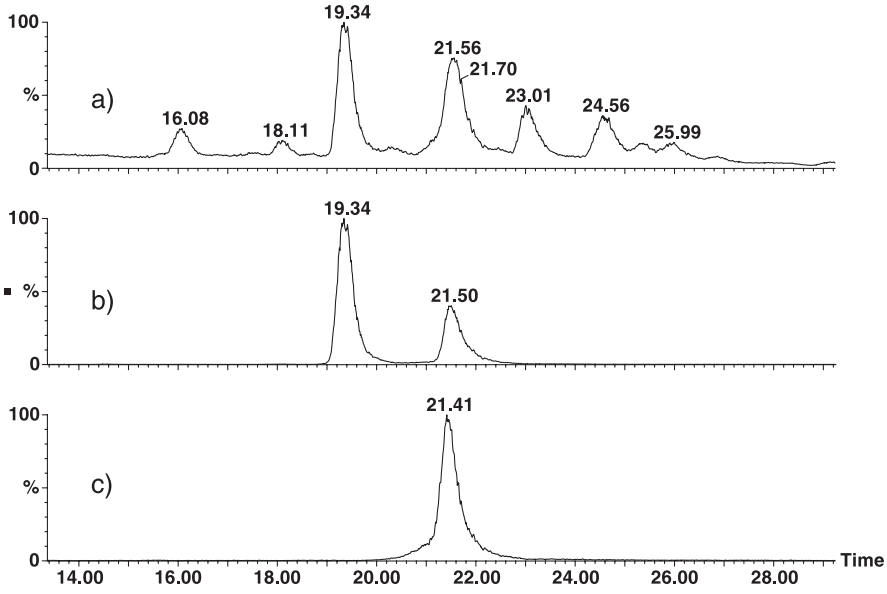

Fig. 3. (a) Total ion chromatogram of the ethyl acetate extract supernatant of a PA14 culture with added labeled PQS. Ion Chromatograms of (b) *m/z* 260 (HQNO and PQS) and (c) *m/z* 264 ions (labeled PQS).

were made and the cells were centrifuged before adding methanol. The pellets were washed with a minimum of fresh culture medium and resuspended with an initial volume of the culture medium containing 50% methanol. The bacterial pellets were found to contain 62.5% (± 9.2) of the PQS while only 26.8% (± 2.6) of the total PQS was in the culture medium. Interestingly, HQNO was almost completely found in the initial culture medium (105.9 ± 9.8%) with only a little part attached to the cells (4.6 ± 0.4%).

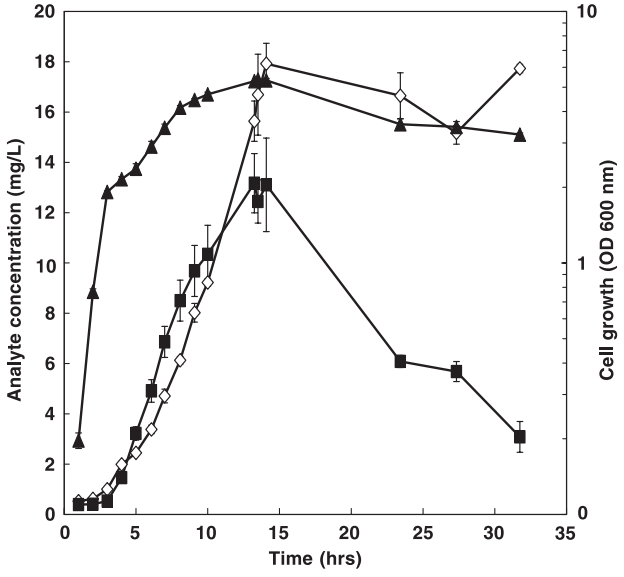

Fig. 4. Concentrations of PQS (■) and of HQNO (◇) in growing cultures of *P. aeruginosa* PA14 (left axis) with incubation time. Cell density (▲) is given in terms of OD at 600 nm (right axis). All measurements were made in triplicate, using the intensity of the corresponding pseudomolecular ion corrected with the internal standard (labeled PQS).

To assess the applicability of the LC/MS method in quantification of PQS and HQNO in actual experimental conditions, the concentration of these compounds was measured in cultures of *P. aeruginosa* PA14 growing in LB medium and compared to the bacterial growth curve (Fig. 4). The graph clearly demonstrates that production of both compounds starts at the end the logarithmic growth phase and their production rate is maximal early during the stationary phase.

We also used our assay to screen various *P. aeruginosa* strains and other *Pseudomonas* species for PQS production in LB medium. The culture media were centrifuged prior to extraction in order to reproduce the initial steps of the protocol developed by Pesci et al. [6]. After 16 h of growth in 6-ml cultures, the concentrations of PQS and HQNO were 4.5 and 9.3 mg/l, respectively, in the supernatant of a PA14 culture. Strain PAO1 produced 4.4 and 8.5 mg/l of PQS and HQNO, respectively. No PQS could be detected in any other *Pseudomonas* strains tested, including *P. aeruginosa* PAK.

4. Discussion

PQS is an intercellular signaling molecule produced by *P. aeruginosa* that is involved in regulating the production of virulence factors. The biological role of this compound is poorly understood and no reliable quantification method was available. The method reported here allows fast and accurate quantification of PQS and HQNO directly in the culture medium without the prior requirement to extract nor to derivatize the sample. The method is simple, requiring only the addition of a methanolic solution of the deuterated PQS internal standard and a centrifugation step to remove

the bacteria and particulate matter. Methanol is also used to overcome the problems associated with the limited solubility of PQS in aqueous solution. We achieved an optimal level of PQS recovery by adding a final concentration of 50% methanol to culture samples before cell removal by centrifugation, but not if the cells were removed before adding the solvent. This indicates that a large proportion of PQS likely binds to bacterial cells and not simply precipitated or attached to the vial. This also explains the differences obtained in PQS concentrations from whole cultures or from the supernatant of centrifuged PA14 cultures, the latter value being close to the solubility limit of PQS in LB medium. The binding of 2-alkyl-4-hydroxyquinolines to cell membranes was also observed by Royt et al. [18]. They isolated 2-nonyl-4-hydroxyquinoline, a longer chain analog of PQS unsubtituted at the 3 position, from a *P. aeruginosa* cell membrane preparation. The structural similarities of this compound with PQS, and the experience performed with cells centrifuged prior to addition of methanol, show that PQS has good affinity for cell membranes. In contrast, HQNO was mostly found in the culture medium.

The total ion chromatogram shown in Fig. 3 reveals additional compounds present in the culture medium and detected by our method. The *m*/*z* values of many of the ions in these chromatographic peaks correspond to families of 2-alkyl-4-hydroxyquinolines such as those detected by Taylor et al. [19] using gas chromatography coupled to MS (GC/MS), some of which partially coelute with PQS. The advantage of using mass spectrometry is that it can be selective to the compounds of a given mass. The only compound interfering with PQS was the isobaric HQNO, which could be separated from PQS using a 38-min gradient. This allows the concomitant quantification of HQNO, a compound first detected in *P. aeruginosa* cultures in 1956 [20]. HQNO prevents the growth of *Staphylococcus aureus* [21], can modulate the virulence of *Vibrio cholerae* [22] and can inhibit the cytochrome systems of prokaryotic and eukaryotic cells [23]. According to Taylor et al. [19], HQNO is particularly difficult to analyze by GC/MS, either directly or after derivatization. In fact, their method involved the reduction of the N-oxide function of HQNO into the corresponding quinoline, followed by derivatization prior to GC/MS analysis. Because the corresponding quinoline was already present in the extract, HQNO quantification could only be performed by substraction from a sample that was not reduced before derivatization.

The production of PQS and HQNO starts at the same time, approximately at the end of the logarithmic growth phase, and their production rates are maximal at the beginning of the stationary phase (Fig. 4). This is typical of secondary metabolites and in sharp contrast with the results reported by McKnight et al. [7] who concluded that PQS production was maximal late in the stationary growth phase. The PQS bioassay they used [7] was performed with a *lasR* mutant of PAO1 (PAO-R1), which does not produce PQS and contains a *lasB'–lacZ* fusion reporter plasmid [6]. They measured the β-galactosidase activity of this reporter strain upon addition of an ethyl acetate extract of the culture to be tested for PQS. This assay is therefore not directly reflecting PQS concentration since it estimates the presence of PQS through the expression of *lasB*, which is also known to be under the control of the *las* and *rhl* quorum sensing systems. Moreover, it is possible that the ethyl acetate extract contains additional compounds besides PQS that might also activate *lasB* expression. McKnight et al. [7] reported the kinetic of PQS production of the PAO1-JP2 mutant strain [24], which does not produce the AHL autoinducers necessary to activate the transcriptional regulators LasR and RhlR. Since an active LasR is required for PQS production, PAO-JP2 was transformed with pECP39, which encodes a constitutively expressed form of LasR. The way in which the PQS production kinetic was determined was thus highly indirect, and we believe that our results reflect more closely the actual situation. However, it is also possible that differences, such as use of an alternate strain of *P. aeruginosa* and different growth media (peptone tryptic soy broth; PTSB), might contribute to explain our contradictory conclusions about the timing of PQS production. Another contributing factor is certainly the small number of sample points in McKnight's experiment, six for a 48-h period, along with the large deviation on each measurement, due to the biological nature of the assay.

Initiation of PQS production at the end the logarithmic growth phase is compatible with the observation made by Pearson et al. [24] that LasR, which is required for the production of PQS, is induced during the last half of the logarithmic growth phase. Interestingly, PQS concentration decreases after the first half of the stationary phase, while the concentration of HQNO remains relatively stable.

The highest concentration of PQS obtained from whole cultures was 13 mg/l. The concentration of PQS in PAO1 culture supernatant was only 4.4 mg/l. This value corresponds to 16 μM, considerably higher than the 6 μM reported previously [6]. This latter value was obtained by measuring the amount of PQS isolated from a PAO1 PTSB culture supernatant. Because of the inevitable losses occurring through the purification procedures, the amount of material isolated could only correspond to a minimum concentration, as mentioned by the authors. In another paper, D'Argenio et al. [12] performed TLC separation of PQS from a PAO1 PTSB culture and quantitated PQS by densitometry. They obtained a value of approximately 1.5 mg/l, again considerably lower than our own results. They obtained similar results using either a culture supernatant or the entire culture. The differences between their results and the yields obtained in the present study do not arise from different intrinsic PQS production by the two strains because in our hands, similar amounts of PQS were recovered from PAO1 and PA14 LB culture supernatants. The most likely explanation probably lies in generally lower cell densities and production rates achieved in PTSB, compared to those obtained with LB growth medium.

Although PQS was found in rather similar concentrations in cultures from the two *P. aeruginosa* strains PAO1 and PA14, it was not detected in culture supernatants of other *Pseudomonas* species, indicating that PQS is specific to *P. aeruginosa*. However, no PQS could be detected in LB cultures of *P. aeruginosa* strain PAK. Interestingly, this strain is known to produce minimal amounts of pyocyanin and other virulence factors (Ref. [25]; S. Beatson, personal communication) controlled by the *rhl* QS system, which is up-regulated by PQS [7]. This phenotype and the inability of this strain to produce PQS are maybe the result of a mutation in the genes responsible for PQS production or those controlling their expression.

In conclusion, the development of a methodology based on direct analysis of culture supernatants with LC/MS has allowed us to determine that PQS is a secondary metabolite of *P. aeruginosa* produced essentially during the early stationary phase of growth. Moreover, the bulk of the PQS produced is not freely soluble in the extracellular milieu but is mostly associated to the surface of the cells.

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