The Pseudomonas aeruginosa quinolone signal (PQS) has an iron chelating activity

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Summary

Virulence factor production and the development of biofilms in *Pseudomonas aeruginosa* have been shown to be regulated by two hierarchically organised quorum sensing systems activated by two types of small acyl-homoserine lactone signal molecules. Recently, a third type of bacterial signal molecule, the Pseudomonas quinolone signal (PQS), has been identified, which positively regulates a subset of genes dependent on the quorum sensing systems. However, the molecular mechanism underlying PQS signalling has remained poorly understood. In this study the global transcriptional profile of *P. aeruginosa* in response to PQS revealed a marked up-regulation of genes belonging to the tightly interdependent functional groups of the iron acquisition and the oxidative stress response. Remarkably, most of the differentially regulated genes, as well as the induction of *rhlR*, could be traced back to an iron chelating effect of PQS. Our results amount to the elucidation of how PQS affects *P. aeruginosa* and have important implications for the understanding of the complex regulatory circuits involved in *P. aeruginosa* gene regulation.

Introduction

Pseudomonas aeruginosa is an ubiquitous bacterium that can be found in nearly any aquatic and terrestrial habitat. Moreover, *P. aeruginosa* is an important opportunistic pathogen that infects humans, animals, insects and even plants (Chastre and Fagon, 2002; Hutchison and Govan, 1999; Lyczak *et al*., 2000). The ecological success of this opportunistic bacterium can be attributed not only to its broad metabolic versatility, but also to its well-regulated release of virulence factors. In many gram-negative bacteria virulence factor production is controlled by an elegant intercellular communication system, known as "quorum sensing" (QS) (Fuqua *et al*., 2001), which is based on the release of soluble communicator molecules (Camara *et al.,* 2002; Fuqua and Greenberg, 1998; Parsek and Greenberg, 2000; Passador *et al*., 1993; Smith and Iglewski, 2003). When the bacterial cell population reaches a certain threshold, the cell metabolites, called "autoinducers", accumulate in the medium and trigger specific cell density-dependent cell functions. In *P. aeruginosa* two autoinducer synthetases (LasI and RhlI) produce two major cell-to-cell acyl-homoserine lactone signals (N-alkanoyl-L-homoserine lactone and N-(3-oxoalkanoyl)-L-homoserine lactone) (Brint and Ohman, 1995; Latifi *et al*., 1995; Ochsner and Reiser, 1995; Pearson *et al*., 1995; Pesci *et al*., 1997). Together with their corresponding transcriptional activator proteins (LasR and RhlR), the autoinducer sysnthetases comprise the two hierarchically organised QS systems *las* and *rhl* (Latifi *et al*., 1996). They control the regulation of virulence factor production in *P. aeruginosa* (de Kievit and Iglewski, 2000; Passador *et al*., 1993; Pearson *et al*., 1997; Pearson *et al*., 2000; Van Delden and Iglewski, 1998; Winson *et al*., 1995) and have been shown to be involved in biofilm formation and development (Davies *et al*., 1998; de Kievit *et al*., 2001; Hassett *et al*., 2002). Recently, a third intercellular signal was identified as a 3,4-(di)hydroxy-alkyl-quinoline (Pesci *et al*., 1999). This Pseudomonas quinolone signal (PQS) apparently provides a link between the *las* and *rhl* systems (McKnight *et al*., 2000). While the production of PQS seems to be enhanced by the *las* system - although it can be produced in the absence

of *lasR* (Diggle *et al*., 2003) - exogenous PQS induces the expression of *lasB* encoding for the major virulence factor LasB elastase and acts by up-regulating the *rhl* QS system. On the contrary, the loss of PQS biosynthesis has been shown to result in the abolition of primarily *rhl*-dependent QS phenotypes despite continued C4-homoserine lactone biosynthesis (Diggle *et al*., 2003).

With the aim of dissecting the various independent regulation levels for many QS induced genes and of evaluating the impact of PQS on the QS circuitry, we performed a transcriptome analysis of PAO1 cultures supplemented with PQS. The transcriptome profile revealed an induction of the iron acquisition systems as well as the oxidative stress response upon PQS supplementation. Moreover, a rapid loss of free iron from cultures was observed on addition of PQS similar to that found using the iron chelating agent 2,2'-dipyridyl, while an ESI/MS analysis of PQS-Fe(III) solutions indicates PQS chelates iron in a 3:1 complex.

Results and discussion

The transcriptional profile of P. aeruginosa in response to PQS

Using the *P. aeruginosa* Affymetrix GeneChip, whole expression profiles were obtained from PQS supplemented and control cultures at early and late logarithmic and at stationary phase of growth, corresponding to incubation times of 5, 11 and 20 h, respectively. A comparison of the gene expression profile of PAO1 grown in medium supplemented with 40 μM PQS revealed a total of 92 genes that were differentially expressed at least at one of the above sampling times compared to control cultures. This represents approximately 1.7 % of the entire genome (Table 1). 66 of these genes were shown to be up-regulated and 26 were repressed.

The most remarkable finding of the global transcriptional profile of *P. aeruginosa* in response to PQS was the induction of siderophore-mediated iron acquisition systems. We found the iron-regulated pyochelin biosynthetic gene cluster (including the *pchDCBA* operon, the pyochelin synthase gene *phcF* and the Fe(III)-pyochelin outer membrane receptor *fptA* gene) was up-regulated at all three time points in bacterial growth. Moreover, *pvdJ*, *pvdA* and *pvdD* encoding a pyoverdine synthetase, and *aprF* and *aprA* encoding alkaline metalloproteinase precursors - previously shown to be up-regulated under iron depletion (Kim *et al.*, 2005) - were induced in the PQS supplemented cultures. In *P. aeruginosa* the siderophores, pyochelin and pyoverdine are under the control of the ferric uptake regulator (Fur) (Hassett *et al*., 1996; Ochsner *et al*., 1995; Ochsner and Vasil, 1996; Prince *et al*., 1993). The main physiological role of Fur is to repress iron acquisition genes under iron sufficiency thus ensuring that iron transport systems are induced by iron restriction. Recently, Fur repressed regulatory small RNAs (Pseudomonas regulatory RNA involving iron, PrrF sRNAs) have been identified in *P. aeruginosa* and their induction has been shown to lead to the rapid loss of mRNA for the iron co-factored superoxide dismutase *sodB,* the succinate dehydrogenase *sdh* and PA4880 a probable bacterioferritin (Wilderman *et al.*, 2004). Intriguingly, these three genes were

demonstrated to be repressed in our transcriptome analysis of *P. aeruginosa* in response to PQS. Moreover, although the regulation of *brfB* (that was strongly down-regulated upon PQS addition) is not affected by the PrrF sRNAs (Wilderman *et al.*, 2004), we found an induced expression of PA4570 which possesses a strong Fur-box in its promoter region and which has been implicated to play a mediatory role in regulation of *brfB* by Fur (Wilderman *et al.*, 2004). Thus, taken together, PQS affects many genes belonging to the Fur regulon.

A second major findng of the transcriptome profile in response to PQS was that - except for the iron co-factored *sodB* and the heme depended *katA* gene - other genes involved in the oxidative stress response were induced upon PQS addition to the growth medium (Table 1). These genes included *ahpC* and *ahpF* encoding for alkyl hydroperoxide reductases, *trxB2* encoding a thioredoxin reductase, the *fagA-fumC-orfX-sodA* operon, and the probable alkyl hydroperoxide reductase encoded by PA0848. From these genes the *fagA-fumC-orfX-sodA* operon contains a Fur box and the expression of both, *fumC* (encoding a fumerase) and *sodA* (encoding a manganese co-factored superoxide dismutase) have been shown to be enhanced in response to iron deprivation (Hassett *et al*., 1997a; Hassett *et al*., 1997b). Moreover, this operon is controlled by QS (Bollinger *et al.*, 2001). The *fumC* encoded fumerase similar to the *sodA* encoded Manganese co-factored superoxide dismutase does not require iron for activity, so that they might replace the iron dependent FumA, FumB and SodB respectively, when iron becomes limited (Vasil and Ochsner, 1999).

PQS induces pyochelin biosynthesis and affects the oxidative stress response in PAO1

To confirm the transcriptome data we monitored pyochelin production in PQS supplemented cultures by using thin layer chromatography (TLC) assays. TLC analysis of pyochelin extracted from bacteria confirmed that PQS enhances the production of the brightly fluorescent pyochelin in the PQS supplemented cultures. This effect could be reversed by the addition of excess iron (Fig. 1).

In addition to the induction of iron acquisition systems the transcriptional profile of *P. aeruginosa* in response to PQS revealed that many genes which significantly contribute to the oxidative stress response were up-regulated. Thus, we tested H_2O_2 susceptibility of cultures grown in the presence or absence of PQS. As shown in Fig. 2 bacteria that were pre-treated with PQS were significantly more sensitive when exposed to H_2O_2 on solid agar, indicating that PQS induces an oxidative stress in the bacteria that can only partly be relieved by the oxidative stress response system.

Correlation of microarray data to previously published P. aeruginosa *transcriptome studies*

The transcriptional response of *P. aeruginosa* to PQS appears to mainly involve the differential expression of genes belonging to the Fur regulon. As expected, a comparative analysis of our study with published transcriptome studies revealed that a high proportion of the genes affected by PQS have previously been shown to be induced by iron limitation (Table 4, supplementary material).

Moreover, a comparison of previous studies on the *P. aeruginosa* response to iron (Palma *et al*., 2003) and iron depletion (Ochsner *et al.*, 2002) respectively with transcriptome studies on the *P. aeruginosa* response to oxidative stress (Chang *et al*., 2005; Palma *et al*., 2004; Salunkhe *et al*., 2005) showed that iron limitation induced not only genes involved in iron acquisition but also several genes involved in the oxidative stress response (many of them regulated by Fur) and vice versa, oxidative stress was shown to result in the induction of genes involved in iron acquisition (Table 4, supplementary material), arguing for a tight interrelation of the Fur regulon and the oxidative stress response. Accordantly, in this study a high proportion of PQS regulated genes had been shown previously to be affected by oxidative stress (Table 1).

PQS has been described to interfere with the hierarchically organised AHL quorum sensing systems *las* and *rhl,* and to induce *rhl* QS dependent genes (McKnight *et al*., 2000). Thus, it

was expected that some of the previously reported AHL-QS regulated genes would also be affected by PQS addition. 14 of the 93 PQS dependent genes have been reported previously to be QS regulated in at least one of the three recent transcriptome studies that map the AHL-QS regulon in PAO1 (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003) 6 PprB dependent genes (Dong *et al*., 2005) and 36 PQS and VqsR (virulence and quorum sensing regulator) dependent genes were found (Juhas *et al*., 2005) (Table 4, supplementary material). Remarkably in a VqsR mutant the PQS biosynthetic genes (Juhas *et al*., 2005) and the pyochelin biosynthetic genes (Juhas *et al*., 2004) were shown to be down-regulated.

Only 20 PQS induced genes have previously been described to be MvfR (multiple virulence factor regulator) dependent (Deziel *et al.*, 2005) although MvfR controls PQS biosynthesis (Table 4, supplementary material). These included 8 encoding hypothetical proteins and the *mexGH* genes, that were reported to influence the production of multiple QS regulated virulence factors (Aendekerk *et al*., 2002) and that have been implicated to play a role in PQS and AHL dependent cell-to-cell communication (Aendekerk *et al*., 2005). The *mexGH* genes are also regulated by the transcriptional regulator SoxR (Palma *et al*., 2005). Whereas SoxR controls the oxidative stress response in *Escherichia coli* (Pomposiello and Demple, 2001), its role in *P. aeruginosa* remains elusive. In addition to the *mexGH* genes, *rsmA,* which acts as a post-transcriptional negative regulator of secondary metabolites (Pessi *et al*., 2001), was among the PQS and MvfR congenerous regulated genes. Moreover, the MvrR controlled *phzS* which is required for phenazine-1-carboxylic acid conversion to pyocyanin, was induced by PQS addition and 4 genes probably encoding for heat shock proteins (*clpB*, *ibpA*, *hslU* and *hslV*). *BfrB* and PA4880 encoding for a probable bacterioferritin were also affected by MvfR and PQS. The reason for the limited consistency of our results and the global gene expression profile of the MvfR mutant seems to be due to the applied experimental conditions. Whereas we analysed the impact of exogenously added PQS to growing *P. aeruginosa* PAO1, Deziel *et al*. (Deziel *et al.*, 2005) compared a MvfR mutant with the wild-type.

The addition of PQS depletes iron from the growth medium due to its iron chelating activity

Since the transcriptome profile of the PAO1 strain grown in the presence of PQS revealed an iron starvation response, we were interested in whether PQS addition had any effect on the iron concentration in the growth medium. Intriguingly, we found that the addition of PQS to the cultures resulted in a rapid depletion of iron from the medium very similar to the iron depletion effect of the chelating agent 2´2-dipyridyl (Fig. 3).

In order to confirm an iron chelating property, PQS (3,4-(di)hydroxy-2-heptyl-quinoline) dissolved in methanol was added to a Fe(III)-sulphate solution. The colour of the solution changed to reddish-pink suggesting the formation of a complex. ESI/MS analysis of this solution afforded the spectrum depicted in Fig. 4A. Signals corresponding to compounds containing two and three PQS molecules bound to Fe(III) were detected. By collison induced dissociation of the Fe(III) complexes (Fig. 4B and 4C), a very high stability of the complex of two PQS anions bound to Fe(III) could be demonstrated (see Fig. 4 for details). 4-Hydroxy-2-heptyl-quinoline lacking the hydroxyl group at position 3 did not form an analogous complex. Stable PQS (3,4-(di)hydroxy-2-heptyl-quinoline)-Fe(III) complexes, partially containing the homologous 3,4-(di)hydroxy-2-nonyl-quinoline- derivative were also isolated from PQS supplemented PAO1 cultures grown until stationary phase in iron replete medium and identified by ESI/MS and subsequent MS/MS analysis (data not shown).

Low iron medium conditions induce the rhl QS system and PQS production

Although no differential in *rhlI/R* transcription was identified by transcriptomics, PQS strongly induced the production of the *rhl* QS dependent secondary metabolite pyocyanin under our experimental conditions (Fig. 5). Thus, as PQS strongly affected the iron concentration in the medium, we were interested in whether the *rhl* QS system was induced in PQS supplemented cultures as a result of iron depletion. We therefore introduced the pMAL.V vector carrying a *lacZ* transcriptional fusion of *rhlR* (obtained by A. Lazdunski) into

PAO1 and determined the ß-galactosidase activity after growth at low and high iron concentrations. Indeed, we found a significant enhancement of the *rhlR* promoter activity in low iron medium (Fig. 6). These results strongly suggest that the induction of the *rhl* QS system in PQS treated *P. aeruginosa* cultures can at least in part be traced back to the PQS effect on *P. aeruginosa* iron homeostasis. Apart from an induced pyocyanin production and *rhlR* transcription the addition of PQS led to a significant enhancement of the endogenous hydroxy-alkyl-quinolone (HAQ) production as determined by thin layer chromatography (TLC) and GC/MS. After 15 and 23 h of incubation a marked HAQ signal differential (4-hydroxy- and 3,4-(di)hydroxy-alkyl-quinoline derivatives, the latter including PQS, predominantly with a 2-heptyl and in smaller amounts with a 2-nonyl side chain (Bredenbruch *et al*., 2005)) was detected in the PQS supplemented PAO1 cultures as compared to controls, that clearly exceeded the initially added concentrations of 40 µM PQS. Whereas the amount of PQS was only about twice of the amount of the PQS in the control cultures (up to 80 µM PQS was detected in the un-supplemented cultures), the PQS precursor substance, 4-hydroxy-alkyl-quinoline, was found to be significantly more induced upon PQS addition (3-5- fold increase). Remarkably, this induction of HAQ biosynthesis was also observed in TLC (data not shown) when the bacteria were grown under low iron conditions, which is consistent with the results of the transcriptome study of Ochsner *et al*. demonstrating an induction of *mvrR* under low iron medium conditions (Ochsner *et al*., 2002).

Conclusion

P. aeruginosa virulence is controlled by complex regulatory circuits involving the interplay of various systems. Apart from the *las* and the *rhl* homoserine lactone dependent QS systems, the MvfR (multiple virulence factor regulator) system is found in *P. aeruginosa*, which regulates PQS biosynthesis and which is intertwined with the homoserine lactone QS systems (McKnight *et al*., 2000). This study provides novel insight into the molecular mechanisms

underlying PQS signalling and demonstrates that well documented effects of PQS can at least in part be traced back to its activity as an iron chelator. As a consequence of the PQS iron chelating activity, the transcriptional profile of PAO1 in response to PQS reflects an iron starvation response, which is accompanied by the up-regulation of genes required for iron acquisition and an oxidative stress response, some of which have been shown to be also regulated by QS. Furthermore, although no *rhlR* induction upon PQS addition could be detected by transcriptomics, addition of PQS to PAO1 cultures clearly enhanced the *rhl* dependent pyocyanin production and, by using *lacZ* fusions, we could demonstrate that the *rhl* QS system was activated under iron limiting conditions. This suggests that PQS affects the *rhl* QS system indirectly as a result of iron depletion. The finding that PQS influences the *P. aeruginosa* iron homeostasis is an important step in the understanding of complex bacterial interactions and endorses the growing evidence that cell-to-cell signalling mediated by the QS systems can also be strongly affected by environmental factors other than cell density. In fact a link between the QS and the iron regulon has been suggested previously (Bollinger *et al*., 2001; Cornelis and Aendekerk, 2004; Hassett *et al*., 1999; Kim et al., 2005; Stintzi *et al*., 1998; Whiteley *et al.,* 1999). The complexity of QS dependent gene regulation has also become apparent from previous observations of additional biological functions of nonenzymatically formed products of homoserine lactones including binding of iron (Kaufmann *et al*., 2005). Moreover, the PQS precursor substance 4-hydroxy-alkyl-quinoline has recently been described to exhibit iron chelating activities although this was not be confirmed by the use of the synthesized compound (Royt *et al*., 2001). We have now shown, that PQS and other 3,4-(di)hydroxy-alkyl-quinoline derivates, but not the 4-(mono)hydroxy-alkyl-quinoline are potent iron chelators. Consequential, when PQS is added to the growth medium PQS depletes iron from the medium so that the bacteria experience iron deprivation and react with an increased expression of the iron acquisition systems, PQS production and *rhlR* expression. Although these results explain previously observed PQS effects, the

biological role of the iron chelating properties remain elusive. It is reasonable to assume that there is a yet to be defined biological function of PQS that warrants further investigations.

Experimental procedures

Bacterial strains, plasmids and culture conditions

P. aeruginosa PAO1 was obtained from the University of Washington Genome Center and was grown in Brain-Heart-Infusion (BHI) medium at 37°C by shaking with or without the addition of 40 μM PQS, respectively. Alternatively, PAO1 was grown in minimal medium (112 mM DL-alaninine, 20 ml glycerol, 0.8 mM K_2HPO_4, 20 mM $MgCl_2$, 100 mM Na_2SO_4, pH 7.0-7.2) (Frank and DeMoss, 1959) supplemented with either 2.5 μM or 25 μM Fe(II) sulfate, respectively. 4-Hydroxy-heptyl-quinoline (HHQ) was synthesized chemically as described previously (Somanathan and Smith K.M., 2004) and used to produce PQS (3,4-(di)hydroxy-heptyl-quinoline) by the procedures described by Pesci *et al*. (Pesci *et al*., 1999). *LacZ* transcriptional fusions of *rhlR* (pMAL.V) were provided by courtesy of A. Lazdunski (Latifi *et al*., 1996). *P. aeruginosa* was grown in BHI medium supplemented with 60 μg/ml tetracycline when required.

RNA extraction, GeneChip hybridization and data analysis

For RNA extraction bacteria were harvested at early logarithmic, late logarithmic and stationary phase of growth. Three independent cultures of PQS-treated and untreated PAO1 each were pooled and the RNA was immediately stabilized with RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). Subsequently two Affymetrix GeneChips were hybridized for each culture condition. RNA isolation, cDNA generation, fragmentation, biotinylation and GeneChip hybridization and analysis were performed according to the Affymetrix guidelines and are conform to the MIAME requirements (Minimum Information About a Microarray Experiment). Data were combined with the latest annotation from the website of the *P.*

aeruginosa PAO1 sequence and the community annotation project provided at www.pseudomonas.com. Genes, which were found in all four pairings defined by the Affymetrix Microarray Suite Software as having significant changes in their signal intensities and where the mean signal differential is at least twofold are listed in Table 1.

Determination of the iron concentration

The concentration of iron in the culture supernatants was determined by spectrophotometric assay using the iron test kit (Merk) according to the instructions of the manufacturer.

Measurement of ß-galactosidase activity

Miller assays were carried out as described previously (Miller JH, 1972). Briefly, 50-200 µl samples of the bacterial culture was removed and added to the reaction mix and vortexed. The reaction mix consisted of 800-950 µl Z buffer, 5 µl 0.1% SDS and 10 µl chloroform all pre-warmed to 30°C. 200 µl of ONPG (4 mg /ml PBS) was added to the reaction and it was incubated until there was a colour change or for 15 min if there was no obvious colour change.

Extraction of extracellular P. aeruginosa hydroxy-alkyl-quinolones (HAQ) metabolites and thin layer chromatography (TLC)

HAQ metabolites were extracted from *P. aeruginosa* broth cultures with dichlormethane. Briefly, the bacterial cultures were extracted with 1 volume of dichlormethane by vigorous shaking. After centrifugation at 2000 g for 15 min the lower organic layer was evaporated. Thin layer chromatography (TLC) was performed using a silica gel 60 F254 TLC plate. The extracted *P. aeruginosa* material was dissolved in methanol and separated by TLC using 95:5 dichloromethane : methanol as a solvent. Fluorescent spots were visualized under UV light and photographed. The two chemically synthesized HAQs were used as standards in TLC.

GC/MS analysis

Hydroxy-alkyl-quinolines (HAQ) which are mainly the 4-hydroxy- and 3,4-(di)hydroxy-alkyl-quinoline derivatives, including PQS, were analyzed after trimethylsilylation (50/50 pyridine + (BSTFA+1% TMC), 70°C, 1h) on a Thermo-Finnigan GCQ ion trap mass spectrometer (Finnigan MAT corp., San Jose, CA) running in the positive ion EI mode equipped with a 30 m DB5 capillary column. Quantification was achieved by spiking the samples with known amounts of PQS (3,4-(di)hydroxy-heptyl-quinoline).

Electrospray Ionization Mass Spectrometry

Approximately 3 µL of the Fe(III) / PQS solution in methanol /water 2:1 (final concentration 10-100 pmol/μL) was applied to a nanospray gold-coated glass capillary placed orthogonally in front of the entrance hole of a QTOF-II instrument (Micromass, Manchester, U.K.). Approximately 1000 V was applied to the capillary, and the ions entering the spectrometer were separated by the time-of-flight analyzer. For MS/MS experiments, parent ions were selected by the quadrupole mass filter and subjected to collision-induced dissociation (CID). Resulting daughter ions were then separated by the TOF analyzer.

H_2O_2 sensitivity assay

The H_2O_2 sensitivity disk assay was adapted from (Hassett et al., 1999). Briefly, PAO1 was grown at 37°C in BHI medium with and without the addition of 40 µM PQS. 100 µL of the bacterial culture was suspended in 3 mL of LB soft agar at 40°C (0.6% (wt/vol) agar), mixed and poured on Columbia blood agar plates. Sterile filter paper disks were placed on the soft solid agar and the disks were spotted with 10 µl of 30% H_2O_2. Plates were incubated at 37°C for 24 h and the diameter of the zone of growth inhibition was measured. All experiments were performed in triplicate.

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Fig. 1. Thin layer chromatography of dichloromethane extracts of bacteria grown in BHI to stationary phase. Lane 1, PAO1 control; lane 2, PAO1 culture supplemented with 40 μM PQS; lane 3, PAO1 culture supplemented with 40 μM PQS and 100 μM Fe(II), lane 4 pyochelin (→, confirmed by ESI/MS).

Fig. 2. Growth inhibition by H_2O_2 as determined by agar diffusion assays. Cultivation of PAO1 in PQS supplemented medium (40 μM) significantly increased the sensitivity towards H_2O_2 (paired t-test; p value < 0.001). The relative zone of inhibition is given as the mean $\pm$ SD of a triplicate.

Fig. 3. Iron concentrations in the bacterial BHI cultures. One representative experiment out of three is shown.

Fig. 4. PQS complexes Fe(III) ions.

A. ESI mass spectrum of PQS complexed to Fe(III) ions.

Daughter ion spectrum of the complex containing three (B) and two (C) PQS molecules. Whereas the former complex eliminates very easily at low collision energies one PQS molecule, but shows no further fragmentation, the latter complex does not eliminate another intact PQS residue, but predominantly fragments after application of considerably higher collision energy under successive cleavage of both alkyl side chains. This unusual (for low energy CID) breaking of non-polarised carbon-carbon bonds is a clear indication of the high stability of the core complex.

Fig. 5. Pyocyanin production in PQS supplemented PAO1 cultures (40 μM) grown for 15 h in BHI versus controls. Results are given as the mean ± SD of a triplicate.

Fig. 6. *RhlR* promoter activity in PAO1 grown under iron limited (2.5 μM Fe(II)) versus non-limiting conditions (25 μM Fe(II)). Relative values are the mean of three independent experiments, with error bars representing the SD of the mean.

528 **Table 1.** Differentially expressed *P. aeruginosa* genes in response to PQS addition

ORF[a]	Gene name	Incubation at time of sampling[b] 5 h	11 h	20 h	Protein description[b]
Iron-regulated genes[d]					
PA0848			**+76.1**		**probable alkyl hydroperoxide reductase**
PA1245			**+4.9**	**+16.2**	**hypothetical protein**
PA1248	***aprF***			**+2.3**	**Alkaline protease secretion outer membrane protein AprF precursor**
PA1249	***aprA***		**+7.3**	**+3.2**	**alkaline metalloproteinase precursor**
PA2384			**+2.9**		**hypothetical protein**
PA2386	***pvdA***		**+4.4**	**+2.6**	**L-ornithine N5-oxygenase**
PA2399	*pvdD*		+2.6		pyoverdine synthetase D
PA2400	*pvdJ*			+9.3	PvdJ
PA2402			+3.9		probable non-ribosomal peptide synthetase
PA2405			**+9.4**		**hypothetical protein**
PA2412			**+3.5**	**+3.0**	**conserved hypothetical protein**
PA2427			+5.7		hypothetical protein
PA3531	***bfrB***	***-50.4***			***bacterioferritin***
PA4218			**+10.1**	**+8.0**	**probable transporter**
PA4220			**+18.4**	**+18.8**	**hypothetical protein**
PA4221	***fptA***	**+10.2**	**+4.2**	**+4.6**	**Fe(III)-pyochelin outer membrane receptor precursor**
PA4222			**+14.3**	**+4.2**	**probable ATP-binding component of ABC transporter**
PA4223			**+6.4**	**+3.8**	**probable ATP-binding component of ABC transporter**
PA4224	***pchG***	**+9.1**	**+8.5**	**+9.9**	**pyochelin biosynthetic protein PchG**
PA4225	***pchF***	**+20.5**	**+39.7**	**+17.2**	**pyochelin synthetase**
PA4226	***pchE***		**+10.3**	**+16.2**	**dihydroaeruginoic acid synthetase**
PA4228	***pchD***	**+24.2**	**+5.2**	**+4.0**	**pyochelin biosynthesis protein PchD**
PA4229	***pchC***	**+18.7**	**+7.3**	**+3.4**	**pyochelin biosynthetic protein PchC**
PA4230	***pchB***	**+9.9**	**+7.9**	**+12.5**	**salicylate biosynthesis protein PchB**
PA4231	***pchA***	**+18.9**	**+7.9**	**+6.0**	**salicylate biosynthesis isochorismate synthase**
PA4467			+5.7		hypothetical protein
PA4468	***sodA***		**+6.0**	**+9.9**	**superoxide dismutase**
PA4469			**+5.6**	**+5.5**	**hypothetical protein**
PA4470	***fumC1***		**+5.2**	**+3.1**	**fumarate hydratase**
PA4471			**+7.1**	**+22.9**	**hypothetical protein**
PA4570				+5.5	hypothetical protein

Genes of the oxidative stress response[e]					
PA0139	*ahpC*		+9.2	+4.2	alkyl hydroperoxide reductase subunit C
PA0140	*ahpF*		+17.0		alkyl hydroperoxide reductase subunit F
PA0200			*+3.1*	*+7.0*	*hypothetical protein*
PA0201				+9.9	hypothetical protein
PA0250			+13.4		conserved hypothetical protein
PA0284			*+8.5*		*hypothetical protein*
PA0449			+6.9		hypothetical protein
PA0848			**+76.1**		**probable alkyl hydroperoxide reductase**
PA0849	*trxB2*		+5.1		thioredoxin reductase 2
PA1174	*napA*		-3.3	-2.6	periplasmic nitrate reductase protein NapA
PA1245			**+4.9**	**+16.2**	**hypothetical protein**
PA1248	***aprF***			**+2.3**	**Alkaline protease secretion outer membrane protein AprF precursor**
PA1249	***aprA***		**+7.3**	**+3.2**	**alkaline metalloproteinase precursor**
PA1902			+3.4		phenazine biosynthesis protein PhzD
PA1999			-3.0		probable CoA transferase subunit A
PA2008	*fahA*		-9.1		fumarylacetoacetase
PA2009	*hmgA*		-2.5		homogentisate 1,2-dioxygenase
PA2146				*+2.4*	*conserved hypothetical protein*
PA2330			+2.3		hypothetical protein
PA2331			*+2.4*		*hypothetical protein*
PA2384			**+2.9**		**hypothetical protein**
PA2386	***pvdA***			**+2.6**	**L-ornithine N5-oxygenase**
PA2405			**+9.4**		**hypothetical protein**
PA2412			**+3.5**	**+3.0**	**conserved hypothetical protein**
PA3032			+2.9		cytochrome c Snr1
PA3237			+34.1	+25.6	hypothetical protein
PA3287			+18.9	+3.0	conserved hypothetical protein
PA3363	*amiR*	-2.1			aliphatic amidase regulator
PA3520				*+2.9*	*hypothetical protein*
PA3531	***bfrB***	***-50.4***			***bacterioferritin***
PA3822		-9.1			conserved hypothetical protein
PA4131			-3.0		probable iron-sulfur protein
PA4141		*+7.3*	*+4.1*	*+5.2*	*hypothetical protein*
PA4217	*phzS*	*+3.7*			*flavin-containing monooxygenase*
PA4218			**+10.1**	**+8.0**	**probable transporter**
PA4220			**+18.4**	**+18.8**	**hypothetical protein**
PA4221	***fptA***	**+10.2**	**+4.2**	**+4.6**	**Fe(III)-pyochelin outer membrane receptor precursor**
PA4222			**+14.3**	**+4.2**	**probable ATP-binding component of ABC transporter**

PA4223			**+6.4**	**+4.2**	**probable ATP-binding component of ABC transporter**
PA4224	***pchG***	**+9.1**	**+8.5**	**+9.9**	**pyochelin biosynthetic protein PchG**
PA4225	***pchF***	**+20.5**	**+39.7**	**+17.1**	**pyochelin synthetase**
PA4226	***pchE***		**+10.3**	**+16.2**	**dihydroaeruginoic acid synthetase**
PA4228	***pchE***	**+24.2**	**+5.2**	**+4.0**	**pyochelin biosynthesis protein PchD**
PA4229	***pchC***	**+18.7**	**+7.3**	**+3.4**	**pyochelin biosynthetic protein PchC**
PA4230	***pchB***	**+9.9**	**+7.9**	**+12.5**	**salicylate biosynthesis protein PchB**
PA4231	***pchA***	**+18.9**	**+7.9**	**+6.0**	**salicylate biosynthesis isochorismate synthase**
PA4236	*katA*	-4.2	+2.4		catalase
PA4377			-3.6		hypothetical protein
PA4468	***sodA***		**+6.0**	**+9.9**	**superoxide dismutase**
PA4469			**+5.6**	**+5.5**	**hypothetical protein**
PA4470	***fumC1***		**+5.2**	**+3.1**	**fumarate hydratase**
PA4471			**+7.1**	**+22.9**	**hypothetical protein**
PA4587	*ccpR*	*-2.2*	*-3.2*		*cytochrome c551 peroxidase precursor*
PA4613	*katB*		+3.4		catalase
PA4655	*hemH*		+12.6		ferrochelatase
PA4811	*fdnH*	-6.5			nitrate-inducible formate dehydrogenase, beta subunit
PA4812	*fdnG*	-2.8			formate dehydrogenase-O, major subunit
PA4880		*-2.6*			*probable bacterioferritin*
PA4881			+4.9	+2.6	hypothetical protein
PA5170	*arcD*		*+2.3*		*arginine/ornithine antiporter*
PA5460			+4.2		hypothetical protein
MvfR regulated genes[f]					
PA0200				*+7.0*	*hypothetical protein*
PA0284			*+8.5*		*hypothetical protein*
PA0905	*rsmA*			+2.6	RsmA, regulator of secondary metabolites
PA0997	*pqsB*		-2.4		
PA2126				*+2.4*	*conserved hypothetical protein*
PA2274			+3.0		hypothetical protein
PA2331			*+2.4*		*hypothetical protein*
PA3126	*ibpA*		+4.8		heat-shock protein IbpA
PA3520				*+2.9*	*hypothetical protein*
PA3531	***bfrB***	***-50.4***			***Bacterioferritin***
PA4141		+7.3	+4.1	+5.2	hypothetical protein
PA4205	*mexG*		+11.4		hypothetical protein
PA4206	*mexH*		+8.2		probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor

PA4217	*phzS*	*+3.7*			*flavin-containing monooxygenase*
PA4542	*clpB*		+5.0		ClpB protein
PA4587	*ccpR*	*-2.2*	*-3.2*		*cytochrome c551 peroxidase precursor*
PA4880		*-2.6*			*probable bacterioferritin*
PA5053	*hslV*		+3.0		heat shock protein HslV
PA5054	*hslU*		+2.5		heat shock protein HslU
PA5170	*arcD*		*+2.3*		*arginine/ornithine antiporter*
Miscellaneous					
PA0359		-2.0			hypothetical protein
PA0721				-2.8	hypothetical protein of bacteriophage Pf1
PA0753		-3.1			hypothetical protein
PA0962			+3.1		probable dna-binding stress protein
PA1582	*sdhD*			-12.4	succinate dehydrogenase (D subunit)
PA2674		-5.5			probable type II secretion system protein
PA2868			+10.3		hypothetical protein
PA3524	*gloA1*	-2.3			lactoylglutathione lyase
PA3815			+3.6		conserved hypothetical protein
PA4263	*rplC*	-2.6			50S ribosomal protein L3
PA4366	*sodB*	-4.0	-3.0	-4.4	superoxide dismutase
PA4430			-7.7		probable cytochrome b
PA4431			-3.0	-3.5	probable iron-sulfur protein
PA4761	*dnaK*		+2.5		DnaK protein
PA4762	*grpE*		+3.1		heat shock protein GrpE
PA5100	*hutU*	-4.6			Urocanase

a. PA numbers are from http://www.pseudomonas.com

b. The ratios represent expression levels in PAO1 cultures supplemented with 40 µM/ml PQS relative to that of the control at specific growth phase time points. Minus (-) sign indicates the value is higher in the control.

c. Proteins as described by http://www.pseudomonas.com.

d. These genes were identified as iron-regulated in previous transcriptome studies (Ochsner *et al*., 2002; Palma *et al*., 2003)

e. These genes were identified in transcriptome studies on the *P. aeruginosa* response to oxidative stress (Chang *et al*., 2005; Palma *et al*., 2004; Salunkhe *et al*., 2005)

f. These genes were identified as MvfR regulated in a previous transcriptome study (Deziel *et al*., 2005)

g. Genes found to be iron- and oxidative-stress regulated are in bold.

h. Genes found to be MvfR and oxidative stress regulated are italics

Fig.1.

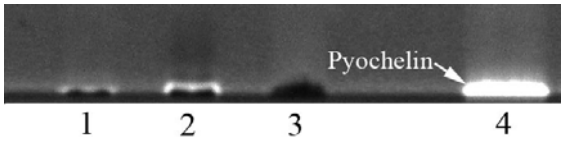


Fig. 2.

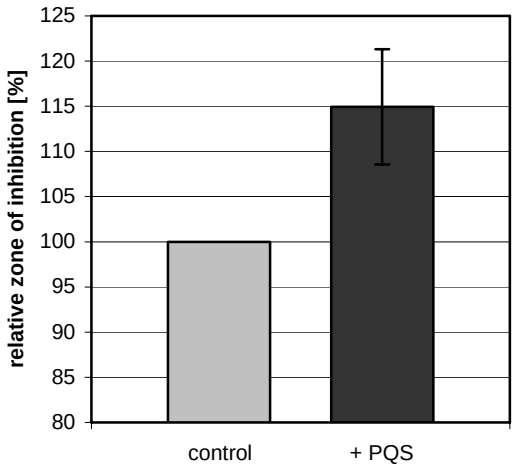
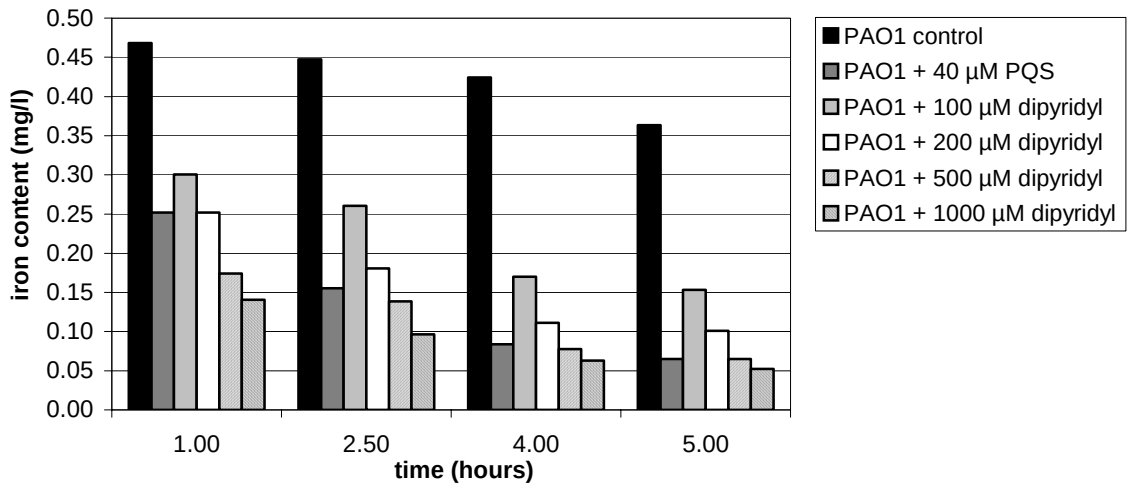


Fig. 3.



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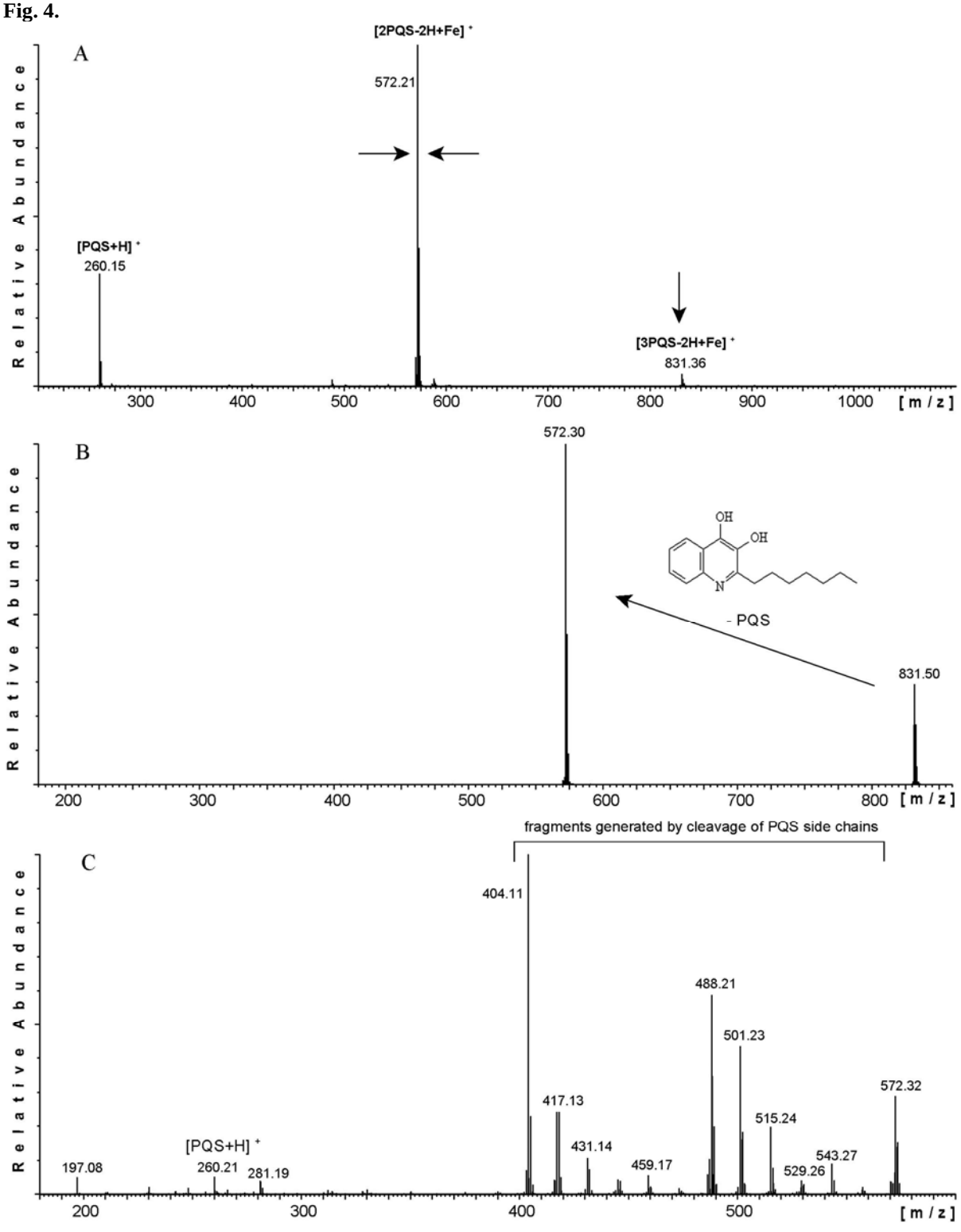
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500
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700
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900
1000
[m / z]
B
572.30
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OH
N
- PQS
831.50
Relative Abundance
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600
700
800
[m / z]
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Fig. 5.

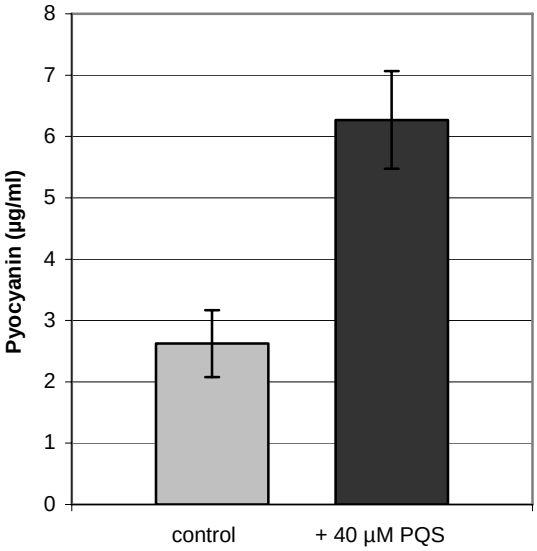
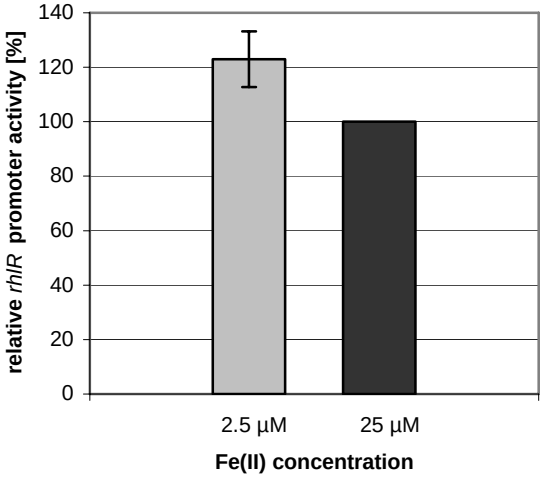

Pyocyanin (µg/ml)
0
1
2
3
4
5
6
7
8
control
+ 40 µM PQS

Fig. 6.


relative rhlR promoter activity [%]
0
20
40
60
80
100
120
140
2.5 µM
25 µM
Fe(II) concentration