

Functional characterization of the gene PA2384 in large-scale gene regulation in response to iron starvation in *Pseudomonas aeruginosa*

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Abstract

The function unknown gene PA2384 of *Pseudomonas aeruginosa* PAO1 has been previously shown dramatically responsive to iron limitation. In the present study, a bioinformatics analysis showed that PA2384 has a weak similarity to the N-terminus DNA-binding domain of Fur, the well-known ferric uptake regulator. To investigate the potential function of PA2384 in iron regulation a *P. aeruginosa* PAO1 recombinant (pUCP20::PA2384) over-expressing PA2384 and a PA2384 disrupted mutant PAO1*PA2384 were constructed. Physiological characterization showed that the knockout mutant had a longer lag phase. Genome-scale transcriptional profiles at different growth stages were compared between the wild type and the Δ PA2384 mutant grown under iron-limiting conditions. The expression of more than 350 genes was affected by the knockout of PA2384. Among them, 71 genes involved in iron uptake were significantly down-regulated in the absence of PA2384. One hundred two quorum sensing (QS) dependent genes displayed differential transcriptions, including genes involved in the biosynthesis of some important virulence factors such as pyocyanin, rhamnolipids and hydrogen cyanide. The transcription of genes responsible for the synthesis of *Pseudomonas* quinolone signal (PQS) was greatly enhanced by the knockout of PA2384. Furthermore, the knockout of PA2384 also resulted in an altered expression of genes involved in electron transfer, central metabolism, phosphorus starvation and translation. It implies that PA2384 might affect more physiological processes than iron acquisition in *P. aeruginosa*.

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1. Introduction

Iron acquisition is crucial to pathogenic bacteria not only because iron participates in many major cellular processes as an essential cofactor, but also because biologically usable iron has a low availability in nature and iron limitation often occurs in mammalian cells. In response, bacteria have evolved several strategies to capture iron. Siderophore-mediated iron transport is one of the most common methods. It is fulfilled via the chelation of extracellular ferric iron with secreted siderophore and subsequent transport of the resulting complex back into the

cells through the outer membrane receptor and inner membrane complex TonB-ExbB-ExbD (Schalk et al., 2004).

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen responsible for many nosocomial infections, produces two typical siderophores, pyoverdine and pyochelin. Pyoverdine comprises a dihydroxyquinoline fluorescent chromophore attached to a specific non-ribosomal synthesized peptide. 23 genes clustered in three loci (PA2385 to PA2402, PA2411-PA2413, and PA2424-PA2428) are responsible for pyoverdine synthesis, uptake and regulation (Ravel and Cornelis, 2003; Smith et al., 2005). PvdS, an alternative sigma factor encoded by PA2426, is supposed to be the main regulator which positively regulates the pyoverdine production and uptake by binding to the iron starvation box (IS box) of the promoter regions of the relevant genes (Visca et al., 2002). Genes involved in the biosynthesis and transport of pyochelin are located on the *P. aeruginosa* genome from PA4218 to PA4231 and their

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expression is activated by the transcriptional regulator PchR (Crosa and Walsh, 2002; Heinrichs and Poole, 1996). The main repressor, ferric uptake regulator (Fur), tightly regulates the siderophore-mediated iron uptake processes to minimize the damage caused by excess iron (Ochsner and Vasil, 1996). Under iron-replete conditions, a Fur dimmer forms a complex with ferrous iron (as a corepressor) and binds to a consensus target sequence, so-called Fur box, in the promoter regions of *pvdS* and *pchR* (Ochsner et al., 1995), thereby blocking their transcriptions and resulting in the subsequent ceasing of the siderophores biosynthesis or uptake. Under iron-deplete conditions, Fur loses its iron cofactor and is released from its binding sites, restoring the transcription of those regulated genes. Apart from its regulatory function in the iron uptake, Fur also plays a role in the expression of genes involved in virulence factors production, oxidative stress response and metabolic pathways. Consequently, it has been proposed to be a global regulator in response to environmental iron concentration (Escobar et al., 1999).

Many virulence factors such as rhamnolipids, elastase, alkaline protease, exotoxin and pyocyanin are also known under the control of quorum sensing (QS) system (Juhás et al., 2005). In *P. aeruginosa*, QS is made up of two interlinked acylated homoserine lactones (AHLs) dependent circuits, the *las* and *rhl* systems, which are further modulated at a third level via the *Pseudomonas* quinolone signal (PQS) system (Juhás et al., 2005). Numerous regulators, such as GacA/GacS, MvaT, Vfr, RpoS, VqsR, MvfR, contribute to the processes at the transcriptional and posttranscriptional levels (Pearson, 2002).

Iron regulation and QS system were suggested to interact with each other. A proteomic analysis found that a *lasI rhlI* double mutant of *P. aeruginosa* exhibited an increased expression of the pyoverdine receptor FpvA and the pyochelin receptor

FptA (Arevalo-Ferro et al., 2003). Juhás et al. (2004) reported in a transcriptome study that the mutation of a virulence and quorum-sensing regulator (VqsR) in *P. aeruginosa* resulted in the down-regulated transcription of *pvdS* gene together with the down-regulation of genes involved in pyoverdine biosynthesis and pyochelin biosynthesis and uptake. Therefore, Cornelis and Aendekerk (2004) postulated that VqsR may connect the QS and iron uptake system in *P. aeruginosa*. More recently, Kim et al. (2005) demonstrated a strong correlation between the expression of the QS regulator LasR and the availability of iron. It is of increasing interest to investigate the link between these two signaling systems.

P. aeruginosa possesses a rather large genome which contains over 6.3 million base pairs and 5570 predicted open reading frames (Stover et al., 2000). According to the recent genome annotation released on 24th January 2007 (<http://www.pseudomonas.com>), more than 40% of the predicted proteins has unknown function. The unknown PA2384 gene locates beside the pyoverdine biosynthesis cluster and it drew our attention because of its remarkably affected expression in response to the availability of iron in a transcriptome analysis (Ochsner et al., 2002). Nevertheless, neither Fur box nor IS box is detected in the promoter region of PA2384. We performed a preliminary bioinformatic survey and found that PA2384 has a weak similarity to the DNA binding domain of Fur. To study the potential role of PA2384 in iron uptake on a genome-wide scale, we constructed a PA2384 over-expressing strain and a PA2384 knockout mutant in this work. Expression profiles of Δ PA2384 mutant of *P. aeruginosa* PAO1 under iron-limiting conditions showed a strongly reduced expression of most iron-responsive genes. We demonstrated that PA2384 may play a role in the positive regulation of iron uptake. In addition to the fact that many QS-controlled genes were affected, the

Table 1
Bacterial strains and plasmids used in this work

Strains/plasmids characteristics	Source/reference	
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild type	Stover et al. (2000)
PAO1-23841	Gm ^R , <i>sacB</i> ⁺ , PAO1 mutant, a plasmid pZP23841 was integrated into the chromosome	This study
PAO1-23842	Gm ^R , <i>sacB</i> ⁻ , PAO1 mutant, the chromosomal PA2384 was replaced by a Gm ^R and GFP cassette	This study
PAO1*PA2384	PAO1 mutant, the chromosomal PA2384 was deleted	This study
<i>E. coli</i>		
Top10	F ⁻ <i>mrcA</i> Δ (<i>mrr-hdsRMS-mcrBC</i>) Φ 80lacZ Δ M15 Δ lacX74 <i>deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
Plasmids		
pUCP20	Ap ^R , cloning vector	West et al. (1994)
pPS858	Ap ^R , Gm ^R , source of the Gm ^R and GFP cassette	Hoang et al. (1998)
pEX18Tc	Tc ^R , <i>oriT</i> ⁺ <i>sacB</i> ⁺ , gene replacement vector	Hoang et al. (1998)
pFLP2	Ap ^R , Flp recombinase-producing vector	Hoang et al. (1998)
pZP23841	Tc ^R , pEX18Tc with a 0.87 kb BamHI-EcoRI fragment containing upstream region of PA2384 gene from PAO1	This study
pZP23842	Tc ^R , pZP23841 with a 0.84 kb BamHI-HindIII fragment containing downstream region of PA2384 gene from PAO1	This study
pZP23843	Tc ^R , Gm ^R , pZP23842 with a 1.8 kb Gm ^R and GFP cassette fragment from pPS858	This study
pUCP20::PA2384	Ap ^R , PA2384 cloned into pUCP20	This study

expression of PQS synthetic genes was triggered on in the very early exponential growth phase in the Δ PA2384 mutant. The present study provides novel insights into the iron-dependent regulatory network and its probable link with the QS system.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

Strains and plasmids used in this study are listed in Table 1. Cells were routinely grown in Luria-Bertani (LB) medium at 120 rpm at 37 °C. When needed, the growth medium was supplemented with antibiotics at following concentrations: for *E. coli*, 100 μ g ml⁻¹ of ampicillin, 10 μ g ml⁻¹ of tetracycline and 10 μ g ml⁻¹ of gentamicin; for *P. aeruginosa*, 200 μ g ml⁻¹ of ampicillin plus 300 μ g ml⁻¹ carbenicillin, and 100 μ g ml⁻¹ of gentamicin. Maintenance media A and B (Mian et al., 1978) and modified glucose minimal medium with 0.6 mg l⁻¹ FeSO₄·7H₂O (Sabra et al., 2002) were used for seed growth and batch cultivation of *P. aeruginosa*. Batch cultivations of strains in a computer controlled bioreactor were carried out as described previously (Kim et al., 2003; Sabra et al., 2002). The control parameters were set as follows: 37 °C, pH 7.0 ± 0.2, pO₂ 10% (v/v), 500 rpm, aeration rate 1 VVM. The initial optical density at wavelength of 600 nm at the time-point of inoculation was controlled at 0.07.

2.2. Biochemical assay

Biomass dry weight was determined as described previously (Sabra et al., 2000). The total extracellular protein in cell-free supernatants was determined by the method of Lowry. Pyocyanin was extracted from culture supernatant and measured as described by Diggle et al. (2003).

2.3. Construction of PA2384 knockout strain

In general, DNA manipulations were performed as described by Sambrook and Russel (2001). Genomic DNA from *P. aeruginosa* was extracted with the blood and cell culture DNA kit (Qiagen).

A PA2384-knockout mutant was constructed by suicide plasmid mediated allelic gene knockout strategy described previously (Hoang et al., 1998). The suicide plasmid was constructed as follows. Two DNA fragments (883 bp and 859 bp), which contained the upstream and downstream region of PA2384 gene, respectively, were amplified by using specific primer pairs of K2384-4F (5'-ACTCGGATCCCATCGTGACGCTCCTTTTCG-3'), K2384-4R (5'-GTGAGAATTCCCGCCAACCATGTCTTCAGC-3') and K2384-5F (5'-TGGACGAAGCTTGACAGGTGGTAATGGTGGGTGG-3'), K2384-5R (5'-CTGGATCCTCTCGGGCAGCGGGTAAAGT-3'), respectively. The resultant upstream fragment of PA2384 was digested with BamHI/EcoRI and then cloned into a BamHI/EcoRI digested suicide vector pEX18Tc, generating plasmid pZP23841. The plasmid pZP23841 was digested with BamHI/

HindIII and then ligated with BamHI/HindIII digested downstream fragment of PA2384, generating plasmid pZP23842. Following the insertion of a Gm^R and GFP cassette from pPS858 in-between of the upstream and downstream region of PA2384 on the plasmid pZP23842, a plasmid pZP23843 harboring a PA2384 knockout mutation was generated in the end. Colony PCR using primer pair C2384-1F (5'-GCTGCAACAGGCTCACCCC-3') and C2384-1R (5'-ACCCGCTACCCACTCGTCC-3') and restriction analysis were done to confirm the structure of each recombinant plasmid. The final recombinant plasmid was electro-transformed into wild type *P. aeruginosa* PAO1. The mutant with integration of suicide plasmid pZP23843 into the chromosome was isolated by gentamicin resistance. The generated strain PAO1-23841 was cultivated in LB broth without gentamicin and then streaked onto LB agar containing 5% (w/v) sucrose and gentamicin. A colony grown on the selective plate was designated as mutant PAO1-23842. The Gm^R and GFP cassette was finally eliminated with the aid of the plasmid pFLP2. The final mutant with complete knockout of PA2384 gene was named as PAO1*PA2384. The generated strains in the procedure are listed in Table 1. The genomic structure of the constructed mutants was verified by colony PCR using the primer pair C2384-1F and C2384-1R. The primer pair Gmfor (5'-CGTTGTGACAATTTACCGA-3') and Gmrev (5'-GATGTTTGTATGTTATGGAGC-3') was used to confirm the existence of Gm^R.

2.4. Construction of a PA2384-overexpressing strain

Two specific primers of 2384-2F (5'-GCTGGATCCCTTTA-CCCCTGCCCCGAGAT-3' with an introduced BamHI site) and 2384-2R (5'-CGGAGAATTCACCATAGAGGCGGCCACAC-3' with an EcoRI site) were used to PCR amplify a 491 bp product harboring the intact PA2384 open reading frame. The chromosomal DNA of *P. aeruginosa* PAO1 was used as DNA template. Then cloning of the PCR fragment into a multi-copy-plasmid pUCP20 resulted in a plasmid pUCP20::PA2384. The recombinant plasmid was further electro-transformed into PAO1, generating a PA2384 overexpressing strain PAO1 (pUCP20::PA2384).

2.5. RNA isolation, microarray hybridization and data analysis

Batch culture samples of *P. aeruginosa* wild type PAO1 and Δ PA2384 mutant were harvested at different growth stages with OD₆₀₀ of 0.2, 1.3 and 2.1 (corresponding to biomass concentrations of 0.17, 0.55 and 0.82 g l⁻¹), respectively. RNA was immediately stabilized with RNAprotect Bacteria Reagent (Qiagen) and total RNA was extracted with RNeasy mini kit following the protocol recommended by the manufacturer (Qiagen). Ten micrograms RNA was processed for microarray analysis according to the manufacturer's manual (Affymetrix). Chip washing and staining was performed with an Affymetrix GeneChip Fluidics Station 400 and chips were scanned with Agilent Genearray Scanner. Experiments were independently performed in duplicate.

Microarray data were analyzed using the software Affymetrix Microarray Suite 5.0. The absolute expression levels were normalized for each chip by globally scaling all probe sets to a target signal intensity of 150. All possible pair-wise array comparisons of replicates were performed with the wild type array as baseline (Chen et al., 2000). Three statistical algorithms (detection, change call, and signal log ratio) were then used to identify differential gene expressions in experimental and control samples. First, transcripts that were absent under both experimental and control conditions were eliminated. Second, if a gene had same change calls in four comparisons (at least three increases or three decreases) and its signal value difference was greater than 100, it was selected for further analysis. Third, the signal log ratio of a differential expression was an average value. A cutoff of log₂ ratio value ≥1 for up-regulated transcripts and ≤−1 for down-regulated transcripts was applied. Only those genes that met all the three criteria above were further analyzed.

2.6. Reverse transcription PCR (RT-PCR) analysis

Total RNA was purified from batch culture samples of both strains taken at OD₆₀₀ 0.2 as described above. cDNA was synthesized using RT Superscript II (Invitrogen) and purified with QIAquick PCR purification kit (Qiagen). To confirm no genomic DNA contamination control groups were prepared following the same procedure except no addition of reverse transcriptase Superscript II. All PCR reactions including control groups were carried out with the Taq polymerase from Qiagen. The gene *pqsA* was selected as representative to verify the gene expression for PQS biosynthesis, RT-PCR was done using primers *pqsA*-F1 (TCGCCGAACAGATTCCCTC) and *pqsA*-R1 (TCAACATGCCCGTTCCTCC). The housekeeping gene *oprL* (Lim et al., 1997) was taken as control using the primers *oprL*-F1 (GACCCGAACGCAGGCTAT) and *oprL*-R1 (GCGACCGGACGCTCTTTA).

2.7. Bioinformatic analysis

Similarity search was done through the basic local alignment search tool (BLAST) (Altschul et al., 1997) server at National Center for Biotechnology Information (NCBI). Ortholog relationship was determined by reciprocal best hit using a local standalone BLAST program suite. Secondary structure analysis and comparison were done by the 3D-PSSM web server (<http://www.sbg.bio.ic.ac.uk/~3dpssm>) (Kelley et al., 2000).

3. Results

3.1. Bioinformatic analysis of the PA2384 gene product

PA2384 is 321 bp in length and encodes a predicted protein with unknown function. Blast search of the deduced amino acid sequence revealed that it is highly conserved in *P. aeruginosa* strains with a 100% identity (*P. aeruginosa* 2192, C3719, PACS2, UCBPP-PA14) or 87% identity (*P. aeruginosa* PA7). Homologues with lower but significant identity (38 to 56%) in other *Pseudomonas* species such as *P. putida*, *P. entomophila*, *P. syringae* pv. *syringae*, *P. fluorescens* and *P. mendocina* were also found. No obvious similarity was found in other non-pseudomonads strains. PA2384 also shows a 35% identity to the N-terminus of the ferric uptake regulator (Fur) (64 of 148 amino acids identical). The predicted secondary structure of PA2384 has a winged helix DNA-binding domain (InterPro entry: IPR009058) (Fig. 1) which is found in Fur and many other transcriptional regulators. A 3-D model generated with the help of the web server “Swiss-Model” further confirmed the high similarity of PA2384 to the N-terminus at 3-D level (data not shown). This raises the possibility that PA2384 may also function as a regulatory protein like Fur. Since PA2384 does not bear the C-terminus domain of Fur responsible for iron-binding and dimerization, it may function in a mechanism different from Fur.

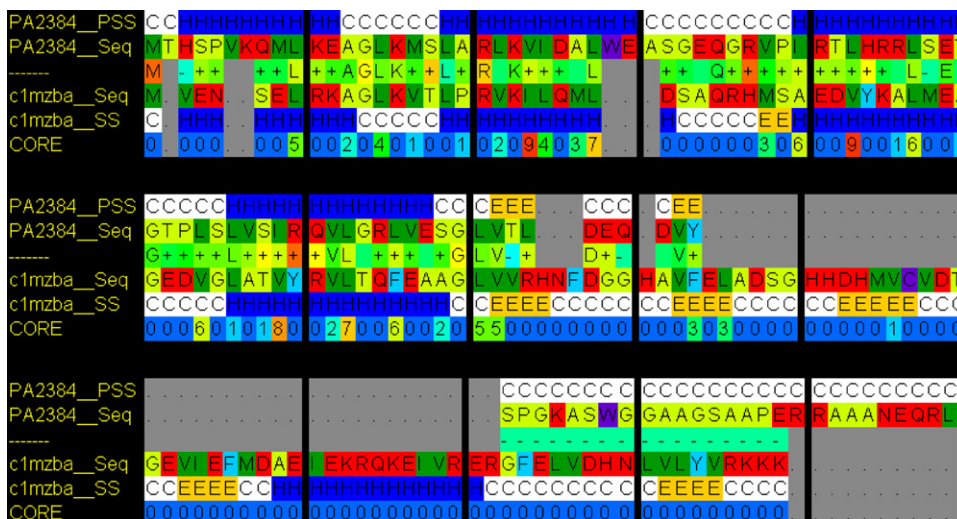


Fig. 1. Alignment of the amino acid sequence and predicted secondary structure of PA2384 in *P. aeruginosa* PAO1 with *P. aeruginosa* Fur protein. PA2384_PSS: predicted secondary structure of PA2384; PA2384_seq: amino acid sequence of PA2384; c1mzba_seq: library sequence of Fur; c1mzba_SS: known secondary structure of the library sequence of Fur; core: a measure of the burial and number of contacts made by a residue. (9 very buried/making many contacts, 0 not buried/making few contacts).

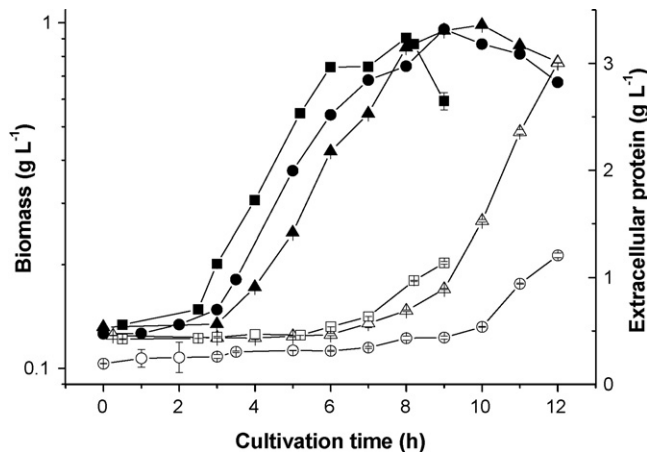


Fig. 2. Comparison of growth and extracellular protein among wild type *P. aeruginosa* PAO1 (●, ○), Δ PA2384 mutant (▲, △) and PA2384-overexpressing strain PAO1 (pUCP20::PA2384) (■, □) in iron deplete medium in batch cultivation.

3.2. Growth comparisons of a Δ PA2384 mutant and a PA2384 over-expressing PAO1 strain (pUCP20::PA2384) with the wild type PAO1

A knockout mutant PAO1*PA2384 was constructed as described in the section Materials and methods. The intact PA2384 gene was disrupted and a scar was left. Additional care was taken to avoid the mutation of the downstream gene PA2383, and the promoter shared by PA2383 and PA2384 was not affected. To elucidate the effect of PA2384 on cellular physiology under iron deficient conditions, we compared the growth characteristics of the mutants with the wild type strain in a computer-controlled bioreactor (Fig. 2). The Δ PA2384 mutant apparently required a longer lag period to adapt to the iron-limiting environment compared with the wild type strain and the over-expression strain PAO1 (pUCP20::PA2384) accelerated the growth process in the lag and initial growth phases. The growth difference supports the positive role of PA2384 in the early response of *P. aeruginosa* to iron starvation.

P. aeruginosa is known to secrete many proteins as virulence factors. Therefore, we also measured the concentration of extracellular proteins in the supernatant (Fig. 2). The maximum extracellular protein concentration in the culture of PAO1 Δ PA2384 mutant was 2.49 folds higher than that of the wild type under the growth conditions tested.

3.3. Gene expression profile of the PA2384 mutant in comparison with the wild type

Gene expressions in the Δ PA2384 mutant at different growth phase were analyzed using Affymetrix genome-scale microarray and compared with the corresponding gene expressions in the wild-type strain. The transcription of more than 370 genes was altered by the knockout of PA2384, of which 28% encode hypothetical proteins.

In the very early exponential growth phase, only eight genes exhibited significant changes (>3 folds difference). The expression of an unknown gene PA2807 was induced while the rest

seven genes (PA0996–1002) of the PQS biosynthetic cluster were repressed in the Δ PA2384 mutant. In the exponential growth phase, about 100 genes displayed more than 2 folds transcription differences. Among them, the expression of 24 genes was changed by more than 5 folds. In the stationary growth phase, more than 300 genes were influenced by the knockout of PA2384 and more than one fourth were up- or down-regulated by more than 5 folds. Thirty-seven genes were identified as being influenced in both phases by the disruption of PA2384. Functional classes of genes whose expressions were affected in the exponential and stationary growth phases are summarized in Fig. 3. The distribution of the affected genes in different function categories seems that PA2384 possibly exerts a pleiotropic effect (direct or indirect) on the gene expression of *P. aeruginosa*.

3.4. Down-regulation of most iron-responsive genes in the Δ PA2384 mutant

To investigate the probable function of PA2384 in iron regulation, genes previously reported to be iron-regulated were first examined and those affected by the knockout of PA2384 are listed in Table 2. In total, 71 iron-responsive genes were affected and 93% of them exhibited a decreased expression pattern in the Δ PA2384 mutant. According to their functions, they were classified into four groups, pyoverdine synthesis and uptake, pyochelin synthesis and uptake, heme uptake and utilization and other iron-regulated genes. Most genes responsible for the biosynthesis of the pyoverdine peptide backbone were affected (see Table 2). PA2424 encoding a non-ribosomal peptide synthetase (PvdL) responsible for the biosynthesis of pyoverdine chromophore (Mossialos et al., 2002) was also down-regulated by 7.85 folds in the Δ PA2384 mutant. Particularly, the expression of *pvdS*, encoding the positive regulator for pyoverdine biosynthesis and uptake, was strongly affected by the knockout of PA2384 (7.25 folds decrease). It implies that PA2384 may have a positive effect on the transcription of pyoverdine biosynthesis genes via the function of PvdS. PA2385 encoding an acylase showed a slight change by 1.5 folds. PA2387 and PA2388, which encode a sigma factor FpvI and an anti-sigma trans-membrane sensor FpvR, respectively (Redly and Poole, 2005) and were supposed to be directly regulated by Fur (Ochsner et al., 2002), were not affected by the knockout of PA2384. However, PA2387-induced transcription of PA2398 (*fpvA*, the pyoverdine receptor gene) was decreased surprisingly by 2.57 folds in the mutant, suggesting that *fpvA* might be also controlled by PvdS as the case in *P. syringae* (Ravel and Cornelis, 2003).

All the 13 genes of two pyochelin biosynthesis operons (*pchDCBA* and *pchEFGHI*) and a ferripyochelin transport operon (*fpABCX*) (Michel et al., 2007) displayed an extremely strong decrease in the mutant, and their corresponding regulatory gene *pchR* has a 2.5 folds decrease. We postulated that PA2384 may activate the pyochelin biosynthesis and uptake via PchR.

The production of ferrisiderophore receptor proteins was reported to be negatively regulated by Fur (Escobar et al., 1999). In our study, all three genes *tonB*, *exbB2* and *exbD2* were found

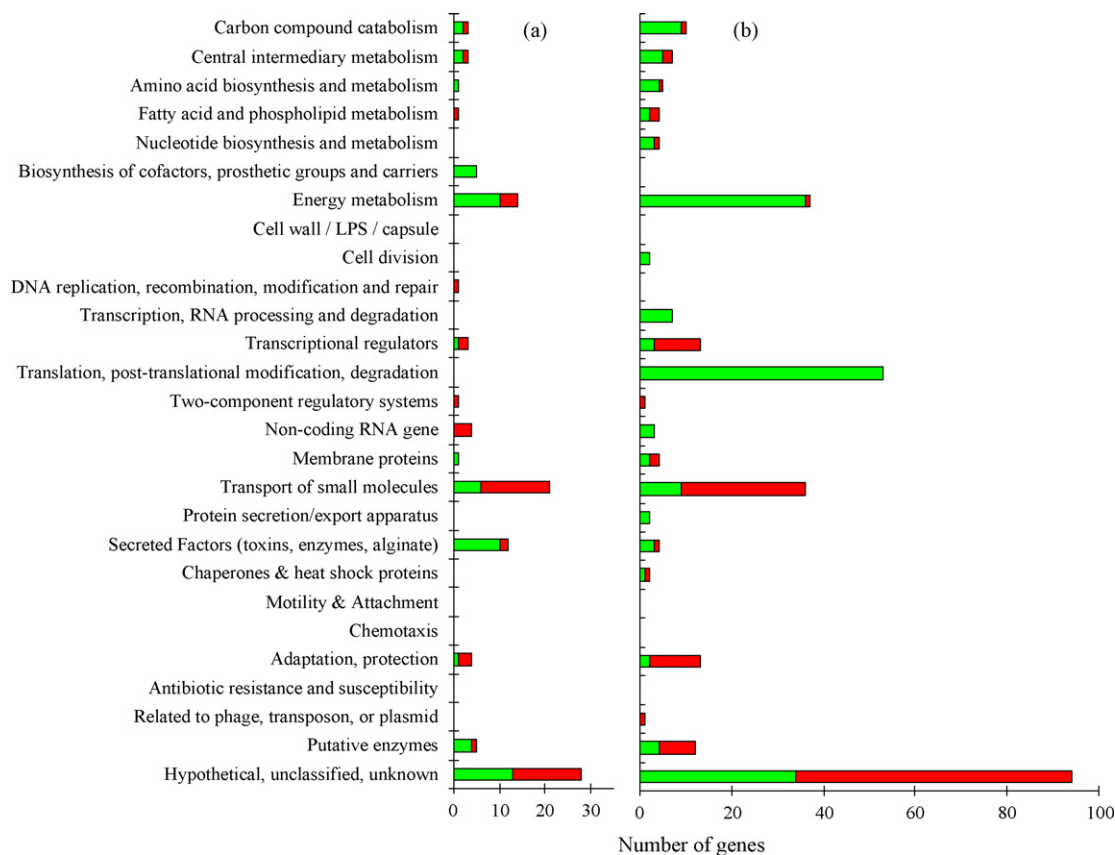


Fig. 3. Functional classes of PA2384-affected genes in *P. aeruginosa*. (a) in the exponential growth phase, $OD_{600} = 1.3$; (b) in the stationary growth phase, $OD_{600} = 2.1$. Genes with at least two-fold change in the knockout mutant PAO1*PA2384 compared to those in the wild type PAO1 are shown. Functional classes are determined according to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>). Green bar and red bar represent numbers of repressed genes and activated genes, respectively.

to be down-regulated in the Δ PA2384 mutant under iron limited conditions (Table 2).

The knockout of PA2384 was also found to affect the expression levels of several Fur-dependent genes involving in heme acquisition and utilization. Genes of two distinct heme-uptake systems *phu* and *has* displayed differential expressions in the Δ PA2384 mutant compared to the parental strain. The *has* locus encodes a high-affinity transport system for hemoglobin-bound heme and consists of a receptor gene *hasR* and a *hasADEF* operon, wherein *hasA* encodes a heme acquisition protein hemophore. The *phu* locus encodes an alternative heme-uptake pathway and consists of *phuR* receptor gene and *phuSTUVW* operon encoding a typical ABC transporter (Ochsner et al., 2000). A 15 folds strong decrease of gene *hasA* and a 2–3.5 folds reduction of *phuR* and *phuST* were detected in the Δ PA2384 mutant. In addition, *hemO* (previously designated as *pigA*) (Ratliff et al., 2001), which is essential in the oxidative degradation of heme to yield free-iron, showed a 10 folds down-regulation in the mutant. Interestingly, two probable sigma-70 factors, PA0675 locating in the same operon of *hemO* and PA3410 locating upstream of *hasR-hasA*, were expressed at a 4 and 2 folds lower level in the mutant.

The excessive uptake of free Fe(II) can be detrimental because iron involves in the formation of highly reactive hydroxyl radicals in combination with oxygen by superoxide

dismutase. *P. aeruginosa* possesses a manganese (Mn)- and iron (Fe)-cofactored superoxide dismutase (SOD) encoded by *sodA* and *sodB*, respectively. The transcription of *fagA-fumC-orfX-sodA* operon, which has been found to be regulated by Fur (Hassett et al., 1997), was 10–20 folds decreased in the PA2384 disrupted mutant compared to the wild type, giving a support for the positive regulatory function of PA2384 in the iron regulation.

Besides the positive regulation shown above, PA2384 also exhibits a negative effect on the expression of some other iron-responsive genes in *P. aeruginosa*. The transcription of five genes was increased in the Δ PA2384 mutant (see Table 2), including *bfrB* and PA4880 (encoding bacterioferritins which function as iron-storage protein) and *sodB* (encoding the iron-cofactored SOD).

3.5. Transcription of multiple QS-dependent genes affected in the Δ PA2384 mutant

The disruption of PA2384 influenced the transcription of 102 QS-dependent genes including those responsible for the production of some important virulence factors such as hydrogen cyanide, rhamnolipids, chitinase, and pyocyanin (see supplementary Table S1).

The most intriguing discovery is perhaps the strong induction of the PQS (3,4-dihydroxy-2-heptylquinoline) system in

Table 2
Iron-dependent genes which were identified to be affected by the knockout of PA2384 during the stationary growth of *Pseudomonas aeruginosa*

PA number	Gene	Function	Fold change ^a
Pyoverdine synthesis and transport			
PA2381		Hypothetical protein	−6.73
PA2383		Probable transcriptional regulator	−6.43
PA2386	<i>pvdA</i>	L-Ornithine N5-oxygenase	−19.03
PA2389		Conserved hypothetical protein	−3.57
PA2392	<i>pvdP</i>	PvdP	−4.68
PA2393	<i>pvdM</i>	Probable dipeptidase precursor	−7.96
PA2394	<i>pvdN</i>	PvdN	−3.77
PA2395	<i>pvdO</i>	PvdO	−2.88
PA2396	<i>pvdF</i>	Pyoverdine synthetase F	−6.73
PA2397	<i>pvdE</i>	Pyoverdine biosynthesis protein PvdE	−7.64
PA2398	<i>fpvA</i>	Ferripyoverdine receptor	−2.57
PA2399	<i>pvdD</i>	Pyoverdine synthetase D	−4.19
PA2400/2401	<i>pvdJ</i>	PvdJ	−11.87
PA2402	<i>pvdI</i>	Probable non-ribosomal peptide synthetase	−5.14
PA2411		Probable thioesterase	−29.78
PA2412		Conserved hypothetical protein	−14.24
PA2413	<i>pvdH</i>	PvdH	−10.55
PA2424	<i>pvdL</i>	PvdL	−7.85
PA2425	<i>pvdG</i>	PvdG	−8.05
PA2426	<i>pvdS</i>	Sigma factor PvdS	−7.25
Pyochelin synthesis and transport			
PA4218	<i>fptX</i>	Probable transporter	−47.76
PA4219	<i>fptC</i>	Hypothetical protein	−17.72
PA4220	<i>fptB</i>	hypothetical protein	−48.80
PA4221	<i>fptA</i>	Fe(III)-pyochelin outer membrane receptor precursor	−22.61
PA4222	<i>pchI</i>	Probable ATP-binding component of ABC transporter	−26.77
PA4223	<i>pchH</i>	Probable ATP-binding component of ABC transporter	−8.99
PA4224	<i>pchG</i>	Pyochelin biosynthetic protein PchG	−12.42
PA4225	<i>pchF</i>	Pyochelin synthetase	−37.59
PA4226	<i>pchE</i>	Dihydroaeruginosic acid synthetase	−85.00
PA4227	<i>pchR</i>	Transcriptional regulator PchR	−2.5
PA4228	<i>pchD</i>	Pyochelin biosynthesis protein PchD	−78.18
PA4229	<i>pchC</i>	pyochelin biosynthetic protein PchC	−61.64
PA4230	<i>pchB</i>	Salicylate biosynthesis protein PchB	−13.47
PA4231	<i>pchA</i>	Salicylate biosynthesis isochorismate synthase	−39.92
Heme uptake and utilization			
PA0672	<i>hemO</i>	Heme oxygenase	−9.64
PA0674	<i>pigC</i>	Hypothetical protein	−3.41
PA0675		Probable sigma-70 factor, ECF subfamily	−4.13
PA3407	<i>hasAp</i>	Heme acquisition protein HasAp	−15.34
PA3410		Probable sigma-70 factor, ECF subfamily	−2.12
PA4708	<i>phuT</i>	Hypothetical protein	−2.00
PA4709	<i>phuS</i>	Probable hemin degrading factor	−3.09
PA4710	<i>phuR</i>	Heme/hemoglobin uptake outer membrane receptor PhuR precursor	−3.52
Other iron-regulated genes			
PA0691		Hypothetical protein	−4.12
PA0692		Hypothetical protein	−3.38
PA0693	<i>exbB2</i>	transport protein ExbB2	−4.71
PA0694	<i>exbD2</i>	Transport protein ExbD2	−2.00
PA0697		Hypothetical protein	−4.88
PA0698		Hypothetical protein	−14.59
PA0699		Probable peptidyl-prolyl cis-trans isomerase, PpiC-type	−3.63
PA0700		Hypothetical protein	−2.58
PA1300		Probable sigma-70 factor, ECF subfamily	−8.24
PA2033		Hypothetical protein	−3.87
PA2452		Hypothetical protein	−10.12
PA3530	<i>bfd</i>	Conserved hypothetical protein	−4.52
PA3531	<i>bfrB</i>	Bacterioferritin	5.44
PA4131		Probable iron-sulfur protein	2.41
PA4134		Hypothetical protein	4.85
PA4366	<i>sodB</i>	Superoxide dismutase	3.46

Table 2 (Continued)

PA number	Gene	Function	Fold change ^a
PA4467		Hypothetical protein	–33.89
PA4468	<i>sodA</i>	Superoxide dismutase	–16.71
PA4469		Hypothetical protein	–17.22
PA4470	<i>funC1</i>	Fumarate hydratase	–9.85
PA4471	<i>fagA</i>	Hypothetical protein	–20.25
PA4570		Hypothetical protein	–10.00
PA4834		Hypothetical protein	–21.68
PA4835		Hypothetical protein	–9.13
PA4836		Hypothetical protein	–4.17
PA4837		Probable outer membrane protein precursor	–10.35
PA4880		Probable bacterioferritin	5.56
PA4896		Probable sigma-70 factor, ECF subfamily	–3.02
PA5531	<i>tonB</i>	TonB protein	–2.61

^a Fold change is defined as the ratio of the expression level of the corresponding genes in the comparison of *P. aeruginosa* knockout mutant PAO1*PA2384 and wild type PAO1. Positive value indicates an increased expression in Δ PA2384 and negative value indicates a decreased expression in Δ PA2384 compared to the wild type PAO1.

Table 3

QPS synthesis genes affected by PA2384 in *P. aeruginosa*

PA number	Gene	Function	Fold change ^a	
			OD = 0.2	OD = 1.3
PA0996	<i>pqsA</i>	Probable coenzyme A ligase	4.95	2.59
PA0997	<i>pqsB</i>	Homologous to beta-keto-acyl-acyl-carrier protein synthase	3.14	2.50
PA0998	<i>pqsC</i>	Homologous to beta-keto-acyl-acyl-carrier protein synthase	16.63	2.57
PA0999	<i>pqsD</i>	3-Oxoacyl-[acyl-carrier-protein] synthase III	4.73	2.62
PA1000	<i>pqsE</i>	Quinolone signal response protein	14.41	NC
PA1001	<i>phnA</i>	Anthranilate synthase component I	3.01	2.41
PA1002	<i>phnB</i>	Anthranilate synthase component II	9.51	NC
PA1003	<i>mvfR</i>	Transcriptional regulator MvfR	NC	NC
PA2587	<i>pqsH</i>	Probable FAD-dependent monooxygenase	NC	NC

Differential expressions of genes involved in the PQS synthesis during different growth phase are shown. All genes shown are not affected by PA2384 in the stationary growth phase (OD = 2.1). PQS, Pseudomonas quinolone signal; NC, no change.

^a Fold change is defined as the ratio of the expression level of the corresponding genes in the comparison of *P. aeruginosa* knockout mutant PAO1*PA2384 and wild type PAO1.

the mutant (Table 3). The transcription of two operons *phnAB* and *pqsABCDE* (encoding enzymes for the biosynthesis of PQS precursors, the primary precursor anthranilate and the direct precursor 4-hydroxy-2-heptylquinoline (HHQ), respectively) (Deziel et al., 2004) were found to be significantly up-regulated at the onset of the exponential growth phase in the Δ PA2384 mutant compared with the wild type. This effect diminished later and disappeared in the stationary growth phase. The differential expression of the PQS biosynthesis genes in the early exponential phase was confirmed with a RT-PCR analysis using *pqsA* as an example (Fig. 4). A TLC analysis of PQS extracted from total cell culture in the very early exponential phase (OD₆₀₀ of 0.2) showed a weak but obvious spot of PQS in the Δ PA2384 mutant, whereas no spot was detected in the wild type (data not shown). The results indicate that PA2384 may repress the synthesis of PQS, or more accurately, the synthesis of HHQ since the transcription of *pqsH* was not affected (*pqsH* encodes a FAD-dependent monooxygenase converting HHQ into the signal molecule PQS). We believe that this effect should not be imposed via the PQS synthesis regulators MvfR and LasR/RhlR (Wade et al., 2005) because all these three regulators were not affected in the present study.

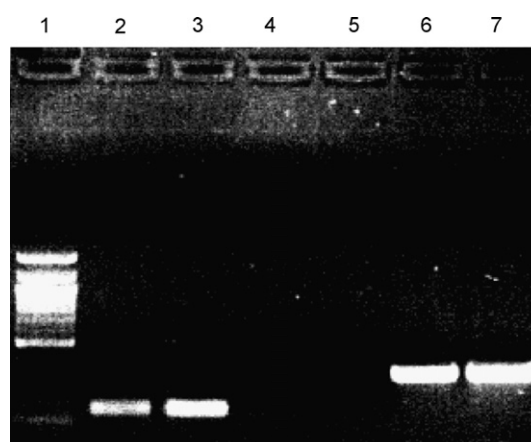


Fig. 4. Confirmation of the differential expression of PQS synthesis gene *pqsA* between *P. aeruginosa* PAO1 and the Δ PA2384 mutant by reverse transcription PCR. Samples were taken at the onset of the exponential growth phase (OD₆₀₀ of 0.2). Lane 1, DNA marker; lane 2, PAO1, primer *pqsA*-F1 and *pqsA*-R1; lane 3, Δ PA2384 mutant, primer *pqsA*-F1 and *pqsA*-R1; lane 4, PAO1 control, primer *pqsA*-F1 and *pqsA*-R1; lane 5, Δ PA2384 mutant control, primer *pqsA*-F1 and *pqsA*-R1; lane 6, PAO1, primer *oprL*-F1 and *oprL*-R1; lane 7, Δ PA2384 mutant, primer *oprL*-F1 and *oprL*-R1.

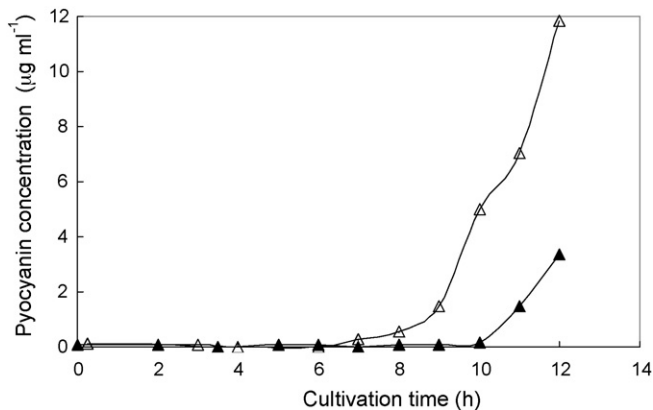


Fig. 5. Enhanced production of pyocyanin in the *P. aeruginosa* ΔPA2384 mutant. The wild type (▲), the ΔPA2384 mutant (△).

The transcription of several PQS-controlled virulence factor genes was also changed by the knock out of PA2384. The *phz* operon (*phzA₁B₁C₂D₂E₂F₂*) and *phzS* involved in the biosynthesis of phenazines and pyocyanin exhibited an up-regulation of 4–10 folds in the mutant in the exponential growth phase but only a slight increase of 1.5–1.98 folds in the stationary growth phase. Biochemical assay confirmed the enhanced production of pyocyanin in the supernatant of the mutant (Fig. 5). Another operon, *mexGHI-opmD*, which is regulated in relation to QS and was recently reported to be essential for cell-to-cell communication via PQS (Aendekerk et al., 2005), was also found to be up-regulated in the exponential and the stationary phases (by 4.5 and 2.6 folds, respectively). The expression of *rhIBA* involved in the rhamnolipids synthesis in the exponential growth phase was increased by 3.7 folds in the mutant. The transcription of *chiC* encoding a chitinase was elevated by 5.7 folds in the stationary growth phase in the mutant.

In the present study, *lasR/lasI* and *rhIR/rhII* encoding the key regulators of QS circuit were not interfered by PA2384. Those PQS-independent but QS-responsive differentially expressed genes might be indirectly affected by PA2384 through other regulators. RpoS, a regulator to modulate the QS system and generally regarded to function in the stationary growth phase (Schuster et al., 2004), is one possible reason because it was found to be differentially expressed at the exponential and stationary stage by the knockout of PA2384.

Taken together, we presume that the effect of PA2384 knockout on some QS-controlled virulence factor genes is more likely realized via PQS/HHQ or RpoS instead of LasR/RhIR.

3.6. Other genes affected by PA2384

Other affected genes besides those involved in the iron regulation and QS system are listed in the supplementary Table S2. They fall into a wide variety of functional categories including energy metabolism, translation, transcription regulators, transport of small molecules and others, implicating that PA2384 may have a global effect. For instance, 37 genes in the class of energy metabolism, encode enzymes responsible for electron transfer, including NADH dehydrogenase, cytochrome *c/o*

oxidase, ATP synthase and enzymes in other energy production pathways. Most of them showed up-regulations in the ΔPA2384 mutant either in the stationary growth phase or at both stages.

Another interesting finding is that several genes associated with phosphate acquisition showed differential transcription in the ΔPA2384 mutant. A large operon of 13-gene for phosphate degradation (*phnPNMLKJIHGFEDC*) showed a significant decreased expression (up to 25 folds) in the stationary growth phase in the mutant. Although most genes in the phosphate limitation responsive Pho regulon and its regulator PhoB were not affected, *phoA* encoding the alkaline phosphatase was shown to be down-regulated by 5 folds in the ΔPA2384 mutant. Our results suggest that PA2384 may have a positive influence on the transcription of those phosphorus starvation-related genes.

4. Discussions

In the present study, a function unknown gene PA2384 was investigated on a genome scale. The blast search showed PA2384 homologues are exclusively present in pseudomonads. The absence of Fur box or an IS box in the promoter region of PA2384 (Ochsner et al., 2002) and the similar spatial structure of PA2384 to the N-terminus of Fur implicate that PA2384 may play an important role in a Fur- or PvdS-independent manner in the iron regulation of pseudomonads.

To elucidate the function of PA2384, we compared the expression profiles of the ΔPA2384 mutant of *P. aeruginosa* PAO1 and the wild type under iron-limiting conditions. In our study, a defined minimal medium and a carefully computer-controlled cultivation system were used to avoid any obscure effects from growth conditions. Moreover, the initial concentration of ferrous ion was kept at only 2 μM instead of the normally used 10–100 μM in order to establish a real low iron condition. Under the conditions studied, Fur is derepressed and regulators such as PvdS and PchR activate the transcription of siderophore biosynthesis genes. However, the present transcriptome analysis showed a significant down-regulation of most genes involved in siderophore biosynthesis and transport in the ΔPA2384 mutant. It suggests that PA2384 may function as an activator under iron limited conditions to promote the expression of the positive regulator PvdS and PchR.

Iron regulation system has been suggested to be linked with QS (Whiteley et al., 1999). A transcriptome analysis of a Tn5::vqsR transposon mutant of *P. aeruginosa* TB showed that the expression of 13 and 14 genes involved in siderophores synthesis and transport, including *pvdS*, was down-regulated in the mutant in the presence of 10% human blood serum and 10 mM H₂O₂, respectively (Juhás et al., 2004). Cornelis and Aendekerk (2004) commented that VqsR could be the bridge connecting the two systems. It is interesting that PA2384 was also down-regulated by the disruption of *vqsR* in the presence of 10% human blood serum. However, in this study, the knockout of PA2384 did not show any effect on the transcription of *vqsR*. Nevertheless, the transcription of many QS-dependent genes was changed in the ΔPA2384 mutant in addition to many iron-responsive genes. PQS, the third level of the QS circuit, was

demonstrated to be affected by the knockout of PA2384. We thereby suggest that PA2384 may link iron uptake and QS system via PQS. The relationship between PQS and iron was found recently. Bredenbruch et al. (2006) reported that PQS bound iron(III). A more recent biophysical analysis revealed that PQS can form a complex with iron(III) at physiological pH at the cell surface. Although exogenous PQS resulted in increased expression of siderophore biosynthesis related genes such as *pvdS* and *pchE* under iron-sufficient conditions (LB medium), it is more likely that PQS functions as a signal to inform the cell the state of iron shortage (Diggle et al., 2007), which derepresses Fur and activates the expression of siderophore biosynthesis genes. Under iron deplete conditions in our study the up-regulation of PQS biosynthesis genes in the PA2384 knockout mutant was associated with a decrease instead of increase in the transcription of siderophore synthesis genes. This may suggest the PQS signaling might require the involvement of PA2384. Further work is needed to investigate the role of PA2384 in the siderophore biosynthesis and its relationship with the PQS signaling.

Another interesting finding is that many genes related to phosphate acquisition showed a remarkable decrease in the PA2384 disrupted mutant. These genes, except the *phn* operon, have been shown to be significantly activated after 12 h of contact of *P. aeruginosa* PAO1 with human airway epithelial (PNHAE) cells (Frisk et al., 2004). *P. aeruginosa* is capable of using phosphonate as phosphorus source by employing the phosphonotase pathway encoded by the *phn* operon (Metcalf and Wanner, 1993). It consists of 13 genes, *phnPNMLKJIHGFEDC*. The homologous operon in *E. coli* consisting of 14 genes has the same gene organization with an additional gene *phnO* between *phnN* and *phnP*. *phnO* was suspected to have a role in the regulation (Metcalf and Wanner, 1993). A *phn* operon identified in *Pseudomonas stutzeri* WM88 (White and Metcalf, 2004) bears no homologue of *phnO*, suggesting that the absence of *phnO* is quite common in pseudomonads and it could have been lost during the evolution.

In summary, the present transcriptome study shows that the knockout of PA2384 resulted in a remarkable down-regulation of iron-responsive genes in the exponential phase and an upregulation of PQS synthesis at the onset of exponential phase under iron limited conditions. It brings new insights into the complex hierarchical network involving iron response and quorum sensing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2007.08.013.

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