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# Novel target genes of PsrA transcriptional regulator of *Pseudomonas aeruginosa*

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**Abstract**

The PsrA transcriptional regulator is involved in stationary phase induced transcriptional regulation of *rpoS* and in negative auto-regulation in *Pseudomonas aeruginosa*. This study was designed to determine whether other loci were regulated by PsrA in *P. aeruginosa*. Computer search was performed of the PsrA binding motif (G/CAAAC $N_{2–4}$ GTTTG/C) against the *P. aeruginosa* genome sequence. Four of 14 analysed promoters responded to and bound PsrA; (i) divergent promoters controlling PA2952/PA2951 and PA2953, (ii) promoter of PA0506 and (iii) upstream region of PA3571. Promoters PA0506 and PA2952–PA2951 were regulated negatively whereas promoters of PA2953 and PA3571 were regulated positively by PsrA. Two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE) analysis on total proteins from *P. aeruginosa* PAO1 and *psrA* knock-out derivative was also performed resulting in the identification of 11 protein spots which were differentially regulated. These studies have indicated PsrA as a global regulator.
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*Keywords:* PsrA regulon; Stationary phase

## 1. Introduction

In their natural environment, bacteria are often challenged by constantly changing nutrient availability and by exposure to various forms of physical stress, including osmotic, oxidative and temperature shock. Exposure to starvation and stresses leads to reduction or cessation of growth, known as stationary phase, resulting in a major switch of gene expression that allows the cell to cope with the new conditions [1]. A very simple and effective mechanism employed by bacteria to bring about such a major switch in gene expression is the use of alternative sigma factors that alter RNA polymerase core specificity [2]. The central regulator during stationary phase in *Pseudomonas* spp., as in other Gram-negative bacteria, is the stationary phase RpoS alternative sigma factor [1,3,4]. In *Escherichia coli*, RpoS regulates more than 100 genes involved in cell survival, cross-protection against various stresses and in virulence [2]. Similarly in *Pseudomonas aeruginosa* RpoS regulates a large set of genes as recently demonstrated using a microarray transcriptome analysis [5]. The levels of RpoS within a bacterial cell are carefully controlled and increase considerably at the onset of stationary phase. The

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regulatory mechanisms governing this control have been extensively studied in *E. coli* revealing that regulation takes place at the level of transcription, translation and post-translational level all responding to various environmental stimuli [1]. Regulation has also been studied to a lesser extent in the fluorescent pseudomonads highlighting that unlike in *E. coli*, transcriptional regulation plays a major role [6]. The global two-component GacA/GacS system and the *N*-acyl homoserine lactone dependent quorum sensing systems are involved in the regulation of *rpoS*; the precise mechanisms of these regulatory controls are unknown and their effect is rather marginal only mildly affecting *rpoS* transcription. We have shown that a TetR family regulator, designated PsrA, plays a major role in positively regulating *rpoS* transcription at the entry of *P. aeruginosa* into stationary phase [7,8]. *psrA* knock-out mutants displayed 90% reduction in *rpoS* promoter activity and 50% in protein levels. DNA-binding studies showed that PsrA binds specifically to the *rpoS* promoter at a sequence −35 to −59 which contains a palindromic motif C/GAAAC $N_{2-4}$ GTTTG/C. In addition, PsrA negatively autoregulates its own expression through binding to a similar sequence in its own promoter [8].

In this study, we identified four new genes, involved in response to stationary phase, regulated by PsrA transcriptional regulator.

## 2. Materials and methods

### 2.1. Strains, plasmids, media and chemicals

The strains used in this study included *E. coli* DH5α [9], *E. coli* pRK2013 [10] and *P. aeruginosa* PAO1 (Holloway collection). *P. aeruginosa* PAO1 and its *psrA* and *rpoS* knock-out mutants have been described previously [7,11]. *E. coli* and *P. aeruginosa* strains were grown in LB medium [12] at 37 °C. The following antibiotic concentrations were used: ampicillin, 100 μg/ml (*E. coli*); kanamycin, 100 μg/ml (*E. coli*) and 300 μg/ml (PAO1); tetracycline, 15 μg/ml (*E. coli*) and 500 μg/ml (PAO1); gentamicin, 100 μg/ml (PAO1). The plasmids used in this study are listed in Table A (Supplementary data). The plasmid transcriptional fusions were constructed as follows. Primers (Table B, Supplementary data) were designed in a way to amplify promoter regions starting from ATG and ending up to 700 bp upstream. Amplified DNA fragments from total genomic PAO1 DNA were treated with *Bam*HI and *Kpn*I restriction enzymes and cloned in pBluescriptKS (or SK) digested with the same restriction enzymes or directly cloned into pBluescriptKS digested with *Sma*I, resulting in pBPA constructs (Table A, Supplementary data). pBPA constructs were sequenced and fragments were then transferred into promoter probe vector pMP220 using different restriction enzymes (*Bam*HI/*Bgl*II and *Kpn*I, *Xba*I and *Kpn*I, *Eco*RI and *Kpn*I) to yield pMPA constructs (Table A, Supplementary data).

### 2.2. Computer analysis

The genome of *P. aeruginosa* PAO1 ([13] http://www.pseudomonas.com) was searched with the FINDPATTERNS (GCG) program using the PsrA binding motif (G/CAAAC $N_{2-4}$ GTTTG/C) derived from alignment of PsrA binding sequence in two promoters known to be regulated by PsrA. However, to investigate the importance of the spacing between the two motifs and to identify a maximum number of candidate genes, we purposely expanded the spacing range to 2–4 nt of the palindromic sequence.

### 2.3. Preparation of total cell proteins and 2-D gel electrophoresis

Total cell proteins were prepared from overnight cultures of *P. aeruginosa* PAO1 and PAO1 *psrA*::Tn*5*. Briefly, 6 mg of wet weight pellet was resuspended in 500 μl of solution [7 M urea, 2 M thiourea, 40 mM dithiothreitol (DTT), 2% w/v 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)] and sonicated 4 × 15 s on ice. For the 2-D analysis, 20 μl (240 μg of total proteins) of sonicated sample was mixed with 230 μl of the same solution to which 1.5 μl of IPG buffer was added before loading. Two-dimensional gel electrophoresis was performed using immobilized pH gradient (pH 3–10 NL, IPG buffers) 13 cm long strips (Amersham Pharmacia Biotech). Strips were rehydrated with entire protein sample for 2 h at room temperature under dry strip-cover fluid (Amersham Pharmacia Biotech). Isoelectric focusing was conducted using IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech) at 20 °C. Proteins were focused for 2 h at 1 kV, 5 h at 5 kV, 3 h at 1 kV, for a total of 30 kV. IPG strips were equilibrated in 50 mM Tris–HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and 15 mM DTT for 20 min at room temperature. Strips were embedded on top of 15 × 15 cm, 12.5% SDS–PAGE gel for the second dimension. Broad Range Prestained Protein Marker (6–175 kDa) purchased from BioLabs was loaded on the same gel at one end of the strip. Protein spots were visualized by Coomassie staining.

### 2.4. Mass spectrometric sequencing and protein identification

The selected protein spots were cut out from the Coomassie Brilliant blue-stained gels and placed in a siliconized microcentrifuge tubes that had been rinsed with

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ethanol, water and ethanol. An internal sequence analysis of the protein spots was performed by using an electronspray ionization mass spectrometer (LCQ DECA XP, ThermoFinnigam). The bands were digested with trypsin, and the resulting peptides were extracted with water and 60% acetonitrile–1% trifluoroacetic acid. The fragments were then analyzed by mass spectroscopy, and the proteins were identified by analysis of the peptides and by using the annotated *P. aeruginosa* genome (www.pseudomonas.com).

### 2.5. Electrophoretic mobility shift

DNA mobility shift assays with purified His6-PsrA were performed with modified previously described procedure [8]. Fragments carrying the promoters of genes coding for acyl CoA dehydrogenase, electron transfer protein, MmsR and MFS transporter were purified from the plasmid constructs with *Bam*HI–*Kpn*I restriction enzymes. Purified DNA (0.1 pmol) was labelled at its *Bam*HI site with the Klenow fragment of DNA polymerase and [α-32P]dCTP. Radiolebeled fragments (1000 cpm) and various quantities of purified His6-PsrA (PsrA protein with six histidine residues at N terminus) (from 0 to 150 ng) were incubated for 30 min at room temperature in 10 μl reaction mixtures containing 1 × binding buffer (20 mM HEPES-KOH pH 7.9, 20% v/v glycerol, 0.2 mM ethylendiaminetetraacetic acid disodium salt (EDTA); 0.1 M KCl, 0.5 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM DTT), 10 μg of bovine serum albumin (carrier protein), 400 ng of salmon sperm (non-specific competitor) DNA and 1.5 mM $MgCl_2$. Supershifting was performed by incubating the reaction mixtures with anti-PsrA antibodies for an additional 15 min at room temperature. Samples were than loaded onto a non-denaturing 4.5% polyacrylamide 0.5 × TBE (44.5 mM Tris, 44.5 mM boric acid, 0.5 mM EDTA) −3% v/v glycerol gel, which was prerun for 1 h at 110 V at room temperature, the samples were also run at 110 V.

### 2.6. Recombinant DNA techniques

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with the Klenow fragment of DNA polymerase, transformation of *E. coli* and SDS–PAGE analysis were performed as previously described [14]. Analytical amounts of plasmids were isolated by procedure described by Birnboim [15], whereas preparative amounts were purified with Qiagen columns (Qiagen, Hilden, D). Total DNA from *Pseudomonas* was isolated by Sarkosyl-pronase lysis [16]. Triparenatal matings from *E. coli* to *Pseudomonas* were performed with an *E. coli* (pRK2013) helper strain as previously described [10].

### 2.7. Reporter gene fusion assays

Transcriptional fusions of all promoters possibly regulated by PsrA were made using pMP220 which harbors a promoterless β-galactosidase *lacZ* gene. β-galactosidase activity was determined by method essentially described by Miller [12] with the modifications of Stachel [17]. Miller units were defined as $OD_{420} \times 1000/OD_{600} \times T(\text{min}) \times V(\text{ml})$. All measurements were done in triplicate and the mean value is given.

## 3. Results

### 3.1. Genomic analysis of the P. aeruginosa PAO1 chromosome for sequences representing potential PsrA binding promoters

Having previously established that the TetR family regulator PsrA was an important positive transcriptional regulator of *rpoS* and a negative auto-regulator via specific DNA-binding to a region of *rpoS* and *psrA* promoters [6–8], it was of interest to determine whether PsrA was transcriptionally regulating other loci in the *P. aeruginosa* genome. The *P. aeruginosa* PAO1 genome was therefore subjected to a degenerate pattern search using the PsrA binding consensus sequences. The subsequence used to search the *P. aeruginosa* genome was SAAAC $N_{2–4}$ GTTTS where S was C or G and 2–4 was the spacing between the two palindromic motifs. This search resulted in the identification of the previously reported *psrA* and *rpoS* binding sites and in 16 new possible PsrA-binding sites distributed randomly on the chromosome of *P. aeruginosa* PAO1 (Table 1). Regions including 600 bp downstream of the potential PsrA binding sites were examined for the presence of open reading frames (ORFs) (Fig. 1 and Table 1). Fig. 1 illustrates the specific region in the chromosome where these putative binding sites were located with respect to which ORF and Table 1 shows the precise location of the putative binding site, the putative DNA-binding sequence and the possible downstream ORF that this PsrA-site might be regulating.

### 3.2. Gene expression analysis of putative PsrA regulated promoters identified by comparative genome analysis

In order to determine whether the putative PsrA binding sites identified using a comparative genome analysis search (see above) represented transcriptionally regulated PsrA-dependent promoters, we tested 14 of them by cloning with adjacent DNA into the *lacZ* wide-host range pMP220 promoter probe vector. These 14 putative binding sites were located in or near intergenic regions and what was believed to be a complete promoter of a putative ORF. Of these, four putative

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Table 1
Predicted binding sites for PsrA in the *Pseudomonas aeruginosa* PAO1 genome and β-galactosidase activity of these promoters

| Binding site | PA number of downstream genes | Position on PAO1 chromosome | Gene/protein | Distance from ATG | β-galactosidase activity (MU) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | WT | *psrA* mutant | *rpoS* mutant | Fold change |
| GAAAC CC GTTTC | PA0413 | 453497–453508 | *pilL* | 618 | 1282 | 1368 | 1315 | NS |
| CAAAC GCCT GTTTG | PA0506 | 564778–564791 | Acyl CoA dehydrogenase | 123 | 4305 | 13,545 | 3912 | 3.15 |
| GAAAC TGAA GTTTC | PA0806 | 883112–883125 | Hypothetical | 652 | 2965 | 2830 | 3201 | NS |
| GAAAC GTAT GTTTC | PA1394 | 1515759–1515772 | Hypothetical | 661 | 2130 | 2270 | 2050 | NS |
| GAAAC CG GTTTC | PA2258 | 2487773–2487784 | *ptxR* | 491 | 1069 | 1171 | 1120 | NS |
| GAAAC CG GTTTC | PA2259 | 2487784–2487773 | *ptxS* | 72 | 1030 | 1213 | 1153 | NS |
| GAAAC CG GTTTC | PA2260 | 2488926–2488937 | Hypothetical | 13 | 1224 | 1228 | 1280 | NS |
| CAAAC TCC GTTTG | PA2673 | 3021019–3021031 | *hplV* | 91 | 1256 | 1320 | 1304 | NS |
| CAAAC AAAC GTTTG | PA2952 | 3312671–3312684 | *etfB* | 202 | 4800 | 9940 | 4950 | 2.1 |
| CAAAC GTTT GTTTG | PA2953 | 3312684–3312671 | Electron transfer flavoprotein–ubiquinone oxidoreductase | 106 | 6650 | 5520 | 6470 | 0.83 |
| GAAAC GTAT GTTTC | PA3006 | 3367686–3367699 | *psrA* | 16 | 3890 | 24,378 | 4015 | 6.3 |
| CAAAC ACTT GTTTG | | 3367699–3367712 | | | | | | |
| GAAAC CGGG GTTTC | PA3571 | 4003410–4003423 | *mmsR* | 309 | 3230 | 2357 | 3206 | 0.72 |
| GAAAC CAGC GTTTC | PA3595 | 4029672–4029685 | MFS transporter | 73 | 1268 | 1231 | 1259 | NS |
| CAAAC TTCC GTTTG | PA3622 | 4059323–4059336 | *rpoS* | 411 | 28,789 | 4190 | 27,630 | 0.15 |
| GAAAC GCCC GTTTC | PA4420 | 4955753–4955766 | Hypothetical | 183 | 2465 | 2716 | 2640 | NS |
| GAAAC CG GTTTC | PA4963 | 5572071–5572082 | Hypothetical | 143 | 3933 | 3753 | 3345 | NS |

NS – not significant, fold change – ratio of promoter activities (MU) in *psrA* mutant versus WT.

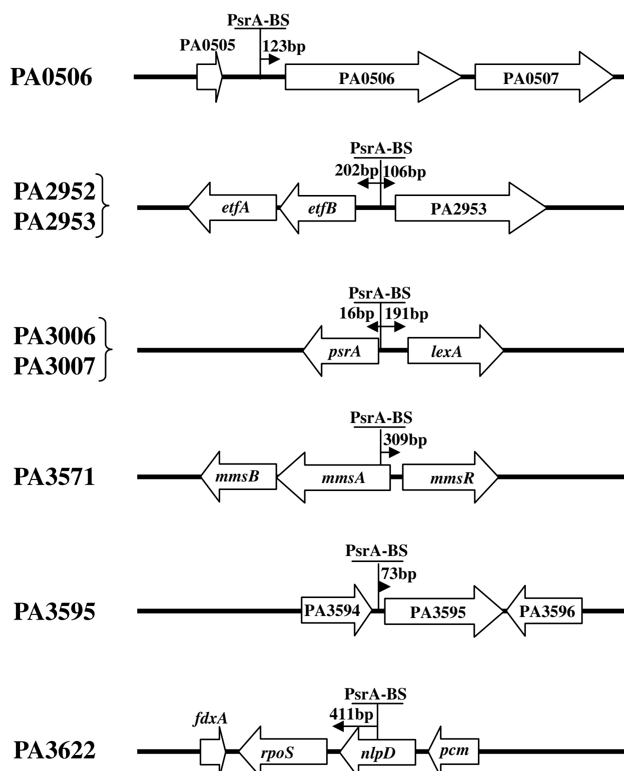


Fig. 1. Location of the putative PsrA binding sites for six promoters regulated by PsrA and for MFS transporter in the *P. aeruginosa* PAO1 genome (for more details see Table 1). These sites were found using a degenerate pattern search against the PAO1 genome. The position of the putative binding site is given as well as its distance to the nearest translation start codon of an annotated ORF. The PA number refers to the possible ORF that PsrA might be transcriptionally regulating (see text for further details). PsrA-BS – PsrA binding site.

binding sites were not tested (PA0099, PA1318, PA5372, PA5451) since they were very distant from the annotated translational start codons and were not in an intergenic region and thus were most probably not located in putative gene promoters. Of the 14 tested putative gene promoters, 4 were shown to be regulated by PsrA since transcriptional fusions were behaving in a PsrA dependent manner (Table 1). These were the promoter of genes PA0506 encoding a probable acyl-CoA dehydrogenase, of operon PA2952–PA2951 encoding an electron transfer flavoprotein β-subunit and α subunit respectively, of PA2953 encoding an electron transfer flavoprotein–ubiquinone oxidoreductase and of PA3571 encoding the transcriptional regulator MmsR. Two of the promoters (PA0506 and PA2952–PA2951) were regulated negatively whereas promoters of PA2953 and PA3571 were regulated positively by PsrA.

### 3.3. Identification of PsrA regulated proteins

In order to characterise PsrA regulated genes more fully in *P. aeruginosa* PAO1 we compared the protein expression pattern in stationary phase of the wild type strain PAO1 versus the PAO1*psrA*::Tn5 mutant by two dimensional (2D) SDS–PAGE gel electrophoresis. Total protein extracts and analysis was performed in triplicate as described in Section 2. The 2D, analysis, revealed differences in protein levels between PAO1 and PAO1 *psrA*::Tn5 mutant in all three experiments in 11 protein spots (Fig. 2). These 11 protein spots were selected for further analysis; proteins present in spots 1, 2, 3, 5, 6, 7, 8, 9 and 10 (electron transfer flavoprotein

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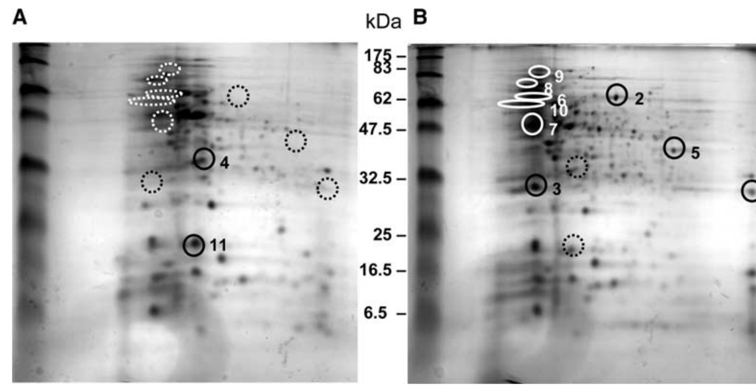


Fig. 2. Comparative 2-D gel analysis of total proteins of *P. aeruginosa* PAO1 (panel A) and *P. aeruginosa* PAO1 *psrA*::Tn*5* (panel B). Numbers of encircled protein spots refer to those represented in Table 2. kDa, kilo Daltons.

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β-subunit; acyl-CoA-dehydrogenase; neomycin-kanamycin phosphotransferase from transposon Tn*5*; fatty acid oxidation complex β-subunit; acyl-CoA-dehydrogenase; isocitrate dehydrogenase and elongation factor Tu; DnaK protein; fatty acid oxidation complex α-subunit and GroEL, respectively) were over-expressed in PAO1 *psrA*::Tn5 mutant, in contrast spots 4 (carabamate kinase) and 11 (conserved hypothetical protein) were more expressed in *P. aeruginosa* PAO1. Peptide mass fingerprinting of tryptic digested fragments was performed on all the 11 protein spots. Each protein spot resulted in the identification of one protein with the only exception of spot 7 which represented two proteins (isocitrate dehydrogenase and elongation factor Tu). Protein spots numbered 2 and 6 contained the same protein, annotated as PA0506, an acyl-CoA dehydrogenase of the same nominal mass of 66 kDa, but different p*I* value, 5.62 (which correspond to calculated p*I* value from aminoacid sequence) for spot 2 and about 4 for spot 6. The difference in p*I* value could be the result of post-translational modifications. The encoding gene for PA0506 contained a functional PsrA binding site in its gene promoter as previously demonstrated (see above). Spot number 3, present only in the PAO1 *psrA*::Tn5 mutant, was the neomycin–kanamycin phosphotransferase from transposon Tn*5*. Protein spot number 1 represented protein PA2952 encoding an electron transfer flavoprotein β-subunit of which the gene, *etfB*, contained a functional PsrA binding site and was shown to be regulated by PsrA (see above). Interestingly, spots 5 and 9 were proteins PA3013 and PA3014 encoded by *faoA* and *faoB* which are organized in an operon involved in fatty acid metabolism. The promoters of all the genes encoding for the identified proteins in this analysis were cloned in the *lacZ* promoter probe vector pMP220 (as described in Section 2) and the expression was determined in strain PAO1, PAO1*psrA*::Tn*5* and PAO1*rpoS*::Tn*5*. The β-galactosidase activities as expected for the two promoters previously identified using a comparative genome search for PsrA binding sites (see above) display PsrA dependent expression. All other gene promoter activities were comparable when obtained in strain PAO1 and the *psrA* knock-out mutant. The promoter activities were also tested in PAO1-*rpoS*::Tn*5* as PsrA is a positive transcriptional regulator of *rpoS*; all promoters displayed comparable activities in PAO1*rpoS*::Tn*5* when compared to wild type PAO1.

### 3.4. Protein–DNA binding studies of PsrA regulated promoters

In order to establish whether the identified PsrA-regulated promoters could bind PsrA, mobility shift assays with the (i) *etfBA* promoter, PA2592/2591 (ii) the pro-

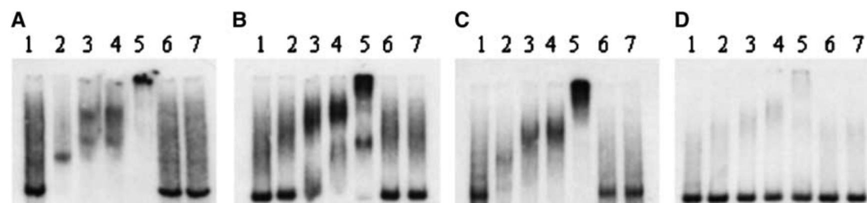


Fig. 3. Retardation of the movement of a DNA fragment containing promoters (composed of complete intergenic region) of acyl CoA dehydrogenase (*Bam*HI-*Kpn*I fragment of 429 bp) [A] electron transfer flavoprotein β-subunit and electron transfer flavoprotein-ubiquinone oxidoreductase (*Bam*HI-*Kpn*I fragment of 377 bp) [B], *mmsR* *Bam*HI-*Kpn*I fragment of 417 bp) [C] and MFS transporter gene (*Bam*HI-*Kpn*I fragment of 251 bp) [D] by purified PsrA protein. The amounts of PsrA protein used were 0, 50, 100 and 150 ng (lines 1–4, respectively) and 150 ng was used with anti-PsrA antibodies (lane 5). A 100-fold excess amount of the same (lane 6) and *psrA* promoter (lane 7) unlabeled DNA fragment was added, except for *mmsR* promoter (100-fold excess amount of the same unlabeled DNA and 10-fold excess amount of unlabeled *psrA* promoter DNA, lane 6 and 7, respectively).

moter of PA0506 (a probable acyl-CoA dehydrogenase), and (iii) the *mmsR* promoter (PA3571), were performed. As a control experiment the promoter of PA3595 (encoding a probable major facilitator superfamily, MFS, transporter) was also used since it contained a putative PsrA binding region however transcriptional studies showed that PsrA had no effect on its transcription (Table 1). The three promoters which displayed PsrA dependent expression were retarded and thus shown to bind PsrA, and the shift was not observed in the presence of excess unlabeled fragment (Fig. 3). A supershift was detected in the presence of anti-PsrA antibodies (Fig. 3). These results confirmed that these gene promoters are regulated by PsrA. The promoter of PA3595 showed no retardation (Fig. 3) confirming the transcriptional fusion data that PsrA was not involved in its regulation.

## 4. Discussion

In this study, several new loci have been found which are regulated at the transcriptional level by the TetR family regulator PsrA of *P. aeruginosa*. PsrA has been originally identified as a positive transcriptional regulator of the stationary phase *rpoS* sigma factor, activating transcription at the onset of stationary phase [7]. In addition it was demonstrated that PsrA acts as a strong negative autoregulator and the binding site in *rpoS* and *psrA* promoters has been determined and was shown to be well conserved [8]. Searching for the PsrA binding motif in the *P. aeruginosa* genome revealed 18 putative binding sites (Table 1 and Fig. 1), however only 4 of the 14 tested were responding and could bind to PsrA as determined with transcriptional fusions and protein–DNA gel retardation assays (Fig. 1, Fig. 3 and Table 1). The search for the PsrA binding site was performed using the consensus, SAAAC $N_{2–4}$ GTTTS, it cannot be excluded that PsrA can bind to variants of this sequence and therefore using this genome search we did not find other functional PsrA binding sites. Alternatively, of the 10 gene promoters tested which contained a putative PsrA binding site but did not display any PsrA dependence, it cannot be excluded that in some other environmental/growth condition these promoters could become PsrA-dependent. Comparing PsrA binding motifs of promoters confirmed to be regulated with PsrA indicate that the functional binding site was C/GAAAC $N_4$ GTTTG/C and that spacing of four nucleotides was important between the two conserved palindromic motifs. Interestingly, the promoter of PA3595, which encodes a major facilitator superfamily (MFS) transporter, contained a perfect GAAAC $N_4$ GTTTC consensus, however we found that it was not regulated and does not bind PsrA in vitro (Table 1, Fig. 3). It could be possible that other sequences are required outside this palindrome or possibly other factors are required for PsrA recognition in certain gene promoters. In summary, we have identified four new loci which are directly regulated by PsrA in addition to the already known *rpoS* and *psrA* promoters. All these promoters have been shown to be able to bind PsrA and have a very well conserved palindromic DNA sequence. In order to identify other PsrA regulated loci, we also performed total 2-D protein analysis of *P. aeruginosa* versus *P. aeruginosa psrA*::Tn*5* and could identify 11 protein spots, out of approximately 300, which were differentially regulated in *psrA*::Tn*5* mutant; two spots were more expressed, eight were less and one was not detectable in PAO1 wild type comparing to PAO1 *psrA*::Tn5 (Fig. 2). The fact that RpoS and PsrA were not identified using this approach indicates that there are probably more proteins which are differentially expressed and were not detected here under these experimental conditions. Interestingly however, three spots represented proteins of which the encoding gene had a PsrA-binding site as found in the comparative genome search and as demonstrated with transcriptional fusion studies and DNA-binding assays (see above). One of these, PA0506 encoding an acyl-CoA dehydrogenase, was detected twice probably due to having different p*I* values possibly because of post-translational modifications. Of the remaining proteins which were differentially expressed, the gene promoter was tested for PsrA dependent transcriptional expression. Surprisingly all promoters, with the exception of PA0506 and PA2592 which contained a PsrA binding site, did not display PsrA dependent transcription in stationary phase in *P. aeruginosa*. The reason for this is not known, however the fact that these protein spots were observed to be differentially regulated in three independent experiments. It could be that PsrA affected the levels of some of these proteins through post-transcriptional and/or post-translational levels of control either directly and/or indirectly. PsrA has been shown to regulate *rpoS* expression in response to stationary phase [6]. A stress encountered by bacteria in stationary phase is starvation for energy-yielding carbon source resulting in the induction of the starvation-stress response [18,19]. Upon induction of this response, numerous structural and physiological changes in the cellular envelope occur in starved cells of Gram-negative enteric bacteria. These include increased lipopolysaccharide in the outer membrane, a shift from phosphatidylglycerol to diphosphatidylglycerol in the inner membrane, decrease in the relative amounts of long-chain monounsaturated fatty acid and increased thickness and cross-linking of the peptidoglycan as well as expanded attachment of the murein layer to the outer membrane [20]. Degradation of these fatty acids through β-oxidation, mediated by acyl-CoA-dehydrogenases, would generate acetyl-CoA to feed the tricarboxylic acid (TCA) cycle, yielding C-compound

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intermediates and electron/H+ ion donors for energy production. This enzyme also catalyses α,β-dehydrogenation of acyl-CoA esters and transfers electrons to an electron transfer flavoprotein via the same mechanism. The acyl-CoA-dehydrogenases (PA0506), the electron transfer flavoprotein (PA2951/PA2952) and electron transfer flavoprotein-ubiquinone oxidoreductase (PA2953) were shown here to be all regulated by PsrA in response to stationary phase and could therefore be part of the same cascade in this process in *P. aeruginosa* linking up these gene products for the first time.

In summary, we have identified new loci regulated by the TetR family regulator PsrA, 4 of which have a functional PsrA box in their gene promoter. PsrA could therefore play an important role in the adaptation to stationary phase.

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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.femsle.2005.04.003 [21].

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