The *Pseudomonas aeruginosa* PsrA responds to long-chain fatty acid signals to regulate the *fadBA5* β -oxidation operon

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β -Oxidative enzymes for fatty acid degradation (Fad) of long-chain fatty acids (LCFAs) are induced *in vivo* during lung infection in cystic fibrosis patients, and this may contribute to nutrient acquisition and pathogenesis of *Pseudomonas aeruginosa*. The promoter region of one *P. aeruginosa* β -oxidation operon, *fadBA5* (PA3014 and PA3013), was mapped. Focusing on the transposon mutagenesis of strain PAO1 carrying the P_{fadBA5} -*lacZ* fusion, a regulator for the *fadBA5* operon was identified to be PsrA (PA3006). Transcriptome analysis of the Δ *psrA* mutant indicated its importance in regulating β -oxidative enzymes. These microarray data were confirmed by real-time RT-PCR analyses of the *fadB5* and *lipA* (encoding a lipase) genes. Induction of the *fadBA5* operon was demonstrated to respond to novel LCFA signals, and this induction required the presence of PsrA, suggesting that LCFAs bind to PsrA to derepress *fadBA5*. Electrophoretic mobility shift assays indicate specific binding of PsrA to the *fadBA5* promoter region. This binding is disrupted by specific LCFAs ($C_{18:1}^{\Delta 9}$, $C_{16:0}$, $C_{14:0}$ and, to a lesser extent, $C_{12:0}$), but not by other medium- or short-chain fatty acids or the first intermediate of β -oxidation, acyl-CoA. It is shown here that PsrA is a *fadBA5* regulator that binds and responds to LCFA signals in *P. aeruginosa*.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen, and the spectrum of infections and diseases it causes is second to none. *P. aeruginosa* can cause infections of the ear, bone, joint, skin and soft tissue, and more serious infections, including meningitis, bacteraemia, endocarditis, ocular infections, hospital-acquired pneumonia, and lung infections in cystic fibrosis (CF) patients (Baltch & Griffin, 1977; Bowton, 1999; Fleiszig *et al.*, 1995; Greenberger, 1997; Lode *et al.*, 2000; Pruitt *et al.*, 1998; Reyes & Lerner, 1983; Richards *et al.*, 1999; Schaberg *et al.*, 1991). *P. aeruginosa* lung infections transpire in nosocomial pneumonia and in CF patients. Nosocomial pneumonia is the

second most common of all nosocomial infections, and *P. aeruginosa* has been the most frequently isolated microbe responsible (Pennington, 1994; Richards *et al.*, 1999). In addition, over 93 % of CF patients between the ages of 18 and 24 have been infected with *P. aeruginosa* (Doring, 1997).

Fatty acid degradation (Fad) pathways may play critical roles in *P. aeruginosa* pathogenesis. It has recently been demonstrated that the Fad pathways of *P. aeruginosa* may have important implications in nutrient acquisition and pathogenesis within the CF lung (Son *et al.*, 2007), through the degradation of an essential lung surfactant component phosphatidylcholine (PC). The degradation of one PC component, long-chain fatty acid (LCFA), potentially occurs through three different β -oxidation pathways, involving three *fadBA* operons (*fadAB1*, PA1737 and PA1736; *fadAB4*, PA4786 and PA4785; and *fadBA5*, PA3014 and PA3013) (Son *et al.*, 2007, 2008; unpublished data). Thus, understanding the genetic regulation of Fad could have direct relevance to the physiology and pathogenesis of this micro-organism within the context of CF infections. The aerobic Fad pathway in *Escherichia coli* is well established, and several of the genes are negatively regulated by FadR in the absence of external fatty acids (FAs) $\geq C_{12}$ (Cronan & Satyanarayana, 1998; DiRusso *et al.*, 1992), including *fadA*, *fadB*, *fadD*, *fadE* and

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Abbreviations: CF, cystic fibrosis; EMSA, electrophoretic mobility shift assay; FA, fatty acid; Fad, fatty acid degradation; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; PC, phosphatidylcholine; SCFA, short-chain fatty acid.

A supplementary table listing sequenced chromosomal transposition sites in strain PAO1- P_{fadBA5} -*lacZ* and a supplementary figure showing the confirmation of three regulatory mutants affecting the expression of *fadBA5* are available with the online version of this paper.

The array data discussed in this paper have been deposited in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE8083.

fadL (Campbell & Cronan, 2002). The *fad* genes are derepressed by acyl-CoA (>C10) binding to FadR, and this complex no longer activates *fab* genes (DiRusso *et al.*, 1992; Henry & Cronan, 1992). However, the regulation of Fad in *P. aeruginosa* has not been characterized, and deciphering the regulation mechanism of Fad is important to further understand the physiology and pathogenesis of *P. aeruginosa*.

In this study, we focused on identifying the regulation mechanism of the *P. aeruginosa fadBA5* operon, which is involved in Fad. We performed transposon mutagenesis to identify a regulator of the *fadBA5* operon. We showed that this regulator, PsaA (PA3006), represses the *fadBA5* operon. This repression is relieved by LCFA signals, as shown by gene fusion data, and this was consistent with our *in vitro* DNA binding results. We performed microarray experiments to further identify other genes within the regulon controlled by this regulatory protein. The microarray data were confirmed by real-time RT-PCR for *fadB5* and *lipA*.

METHODS

Bacterial strains, media, and culture conditions. *E. coli* HPS1 (Schweizer, 1994) was routinely used as a strain for cloning and was cultured in Luria–Bertani (LB) medium (Difco). *P. aeruginosa* strain PAO1 (Holloway *et al.*, 1994; Stover *et al.*, 2000) and derivatives were cultured on *Pseudomonas* Isolation Agar (PIA; Difco) or LB medium. All chemicals were purchased from Sigma. FA (butyric acid, C_{4:0}; N-caproic acid, C_{6:0}; caprylic acid, C_{8:0}; capric acid, C_{10:0}; lauric acid, C_{12:0}; myristic acid, C_{14:0}; palmitic acid, C_{16:0} and oleic acid, C_{18:1}^{Δ9}) stock solutions at 3 % (w/v) were made with equimolar KOH and 1 % Brij-58 [poly(oxyethylene) cetyl ether]. Growth curves (Fig. 1) were performed in 1 × M9 (containing 0.5 mM MgCl₂ and 0.02 mM CaCl₂) + 1 % Brij-58 supplemented with 20 mM glucose or 0.2 % of different FAs as sole carbon source. Growth curves for β-galactosidase activity (Fig. 3) were performed in 1 × M9 + 1 % Brij-58 + 40 mM glucose with or without 0.1 % of the particular FA. Complementation of the *psaA* mutation (Fig. 5b) was performed in LB + 1 % Brij-58 with or without 0.2 % C_{18:1}^{Δ9}. Unless indicated otherwise, the cultures were grown at 37 °C with a shaking speed of 250 r.p.m.

Molecular methods and reagents. Restriction enzymes, dNTPs, T4 DNA polymerase and ligase were purchased from New England Biolabs and used as recommended by the supplier. Plasmids and DNA gel bands were isolated using the Zippy Plasmid Miniprep Kit I and Zymoclean Gel DNA Recovery kit, respectively, from Zymo Research Corporation. Chromosomal DNA was isolated with the IsoQuick nucleic acid extraction kit (Orca Research). *E. coli* competent cell preparations, blunt-ending of DNA fragments with T4 DNA polymerase, and other molecular techniques were performed according to Sambrook & Russell (2001). Oligonucleotide primers (Table 1) were synthesized by Integrated DNA Technologies (IDT). Southern hybridization analysis was performed as described previously (Hoang *et al.*, 1998). *P. aeruginosa* competent cells were prepared as described elsewhere (Choi *et al.*, 2006). Generally, we performed the various PCRs by initial denaturation for 1 min at 94 °C and 34 cycles of 45 s at 94 °C, 30 s at 58 °C, and 1 min kb⁻¹ at 72 °C, and a final step of 10 min at 72 °C was included. *Pfu* was purchased from Stratagene.

Characterization of the PAO1-Δ*fadBA5* mutant strain and complementation study. The PAO1 Δ*fadBA5* mutant strain,

PAO1-Δ*fadBA5*, has been described previously (Son *et al.*, 2007). Complementation of the PAO1-Δ*fadBA5* mutant was done as follows. The *fadBA5* operon, including its native promoter region, was amplified from the PAO1 chromosome by PCR using oligos #287 and #273. The PCR product was digested with *EcoRI* and cloned into mini-CTX2 (Hoang *et al.*, 2000) digested with *SmaI* + *EcoRI*, yielding mini-CTX2-*fadBA5*. Next, mini-CTX2-*fadBA5* was transformed into the PAO1-Δ*fadBA5* mutant to integrate into its chromosome and complement the Δ*fadBA5* mutation.

Growth curve studies were performed on three strains (PAO1, PAO1-Δ*fadBA5* and PAO1-Δ*fadBA5*/miniCTX2-*fadBA5*). These strains were grown overnight at 37 °C in PIA medium. Overnight cultures were washed twice with one volume of 1 × M9 buffer and resuspended in an equal volume of the same buffer. Resuspended cultures were then diluted 100-fold into fresh 1 × M9 + 20 mM glucose or 0.2 % of a FA (C_{4:0}; C_{6:0}; C_{8:0}; C_{10:0}; C_{12:0}; C_{14:0}; C_{16:0} or C_{18:1}^{Δ9}). Growth was then initiated. At each time point, aliquots of each culture were diluted fourfold in 4 % Brij-58 and OD₅₄₀ measurements were taken.

Promoter mapping. We mapped transcript start sites for *fadB5* (PA3014), according to a method for mapping eukaryotic transcript start sites described elsewhere (Tabansky & Nurminsky, 2003). Cells, grown in 1 × M9 + palmitate, were harvested for promoter mapping in exponential phase (OD₅₄₀ ~0.6), early stationary phase (10 h after exponential phase), and late stationary phase (18 h after early stationary phase). All reagents required for transcriptional start-site mapping were supplied in the SMART RACE cDNA Amplification kit (Clontech, BD Biosciences). Briefly, total mRNA was isolated with an on-column DNase I treatment, using the Qiagen RNeasy mini kit. A 1 μg sample of total mRNA was assembled with a SMART primer and the first gene-specific primer (primers #244 and #279, Table 1); each primer was at 2 μM final concentration. cDNA synthesis was carried out with the reagents supplied and as recommended by the manufacturer. The diluted cDNA was used as a template in a PCR with a second nested gene-specific primer (Table 1, primer #280) and the SMART RACE primer. PCR products were purified from gel slices and sequenced with a third nested gene-specific primer (Table 1, primer #281) at our local core facility.

***P_{fadBA5}-lacZ* fusion construction and β-galactosidase assay.** The *P_{fadBA5}-lacZ* transcriptional fusion was constructed by amplifying the *fadBA5* promoter region, encompassing nucleotides 148–266 upstream of the start codon. PCR was performed using primers #287 and #289 with PAO1 chromosomal DNA. The resulting 120 bp fragment was cut with *HindIII* and cloned into *HindIII*- and *SmaI*-digested pTZ120 (a derivative of pTZ110) (Schweizer & Chuanshuen, 2001) to yield pTZ120-*P_{fadBA5}*. For integration into the *P. aeruginosa* genome, the *P_{fadBA5}-lacZ* fusion was removed by *AflIII* digestion, blunt-ended, and then digested with *HindIII*. This fragment was then subcloned between the *HindIII* and *SmaI* sites of mini-CTX2 to yield mini-CTX2-*P_{fadBA5}-lacZ*. Chromosomal integration of this mini-CTX2-*lacZ* fusion vector in *P. aeruginosa*, excision of unwanted plasmid sequences and verification of insertion at the chromosomal *attB* locus were performed as described previously (Hoang *et al.*, 2000).

β-Galactosidase activities were measured for the integrated *P_{fadBA5}-lacZ* fusion under various growth conditions. Cells harbouring the fusions were grown overnight in PIA medium. Overnight cultures were washed twice with one volume of 1 × M9 buffer and resuspended in an equal volume of the same buffer. Resuspended cultures were then diluted 100-fold into fresh 1 × M9 + 40 mM glucose ± 0.1 % FA (C_{4:0}; C_{6:0}; C_{8:0}; C_{10:0}; C_{12:0}; C_{14:0}; C_{16:0} or C_{18:1}^{Δ9}). Growth curves were determined for each culture by diluting them fourfold in 4 % Brij-58, after which OD₅₄₀ was measured. All growth curves had identical growth rates (data not shown). Cell cultures (1 ml) were taken at mid-exponential phase (OD₅₄₀ ~1.4) during the growth curve experiments, and β-galactosidase assays were

Fig. 1. Growth curves of the *fadBA5* mutant and the complemented strain. The PAO1-Δ*fadBA5* mutant and the complemented strain PAO1-Δ*fadBA5*/miniCTX2-*fadBA5*, along with wild-type PAO1, were grown in 1× M9 minimal media supplemented with 20 mM glucose alone (a) or individually with 0.2% different FAs as sole carbon source: C_{4:0} (b), C_{6:0} (c), C_{8:0} (d), C_{10:0} (e), C_{12:0} (f), C_{14:0} (g), C_{16:0} (h) and C_{18:1}^{Δ9} (i). The y axes show OD₅₄₀ and the x axes show time in hours. The growth rates and overall cell densities were identical between the wild-type PAO1 strain and the *fadBA5*-complemented strain. The PAO1-Δ*fadBA5* mutant grew similarly to the wild-type on glucose and C_{4:0} to C_{10:0}. However, compared with PAO1, the PAO1-Δ*fadBA5* mutant showed growth defects (slower growth and lower final cell densities) when grown on C_{12:0} to C_{18:1}^{Δ9} as sole carbon source.

performed from the same cultures. β-Galactosidase assays were done in triplicate and measured in Miller units (Miller, 1992; mean ± SEM).

Identification and characterization of transposon mutants affecting *fadBA5* expression. The fusion strain PAO1-P_{*fadBA5*}-*lacZ* was subjected to transposon mutagenesis using the mariner transposon vector pBT20. The transposon in pBT20 was conjugally transferred by biparental mating into PAO1-P_{*fadBA5*}-*lacZ*, following a protocol described elsewhere (Kulasekara *et al.*, 2005). Mating mixtures were scraped and resuspended in 3 ml LB. Then, 300 μl of this suspension was plated onto PIA + gentamicin (Gm) at 150 μg ml⁻¹ + X-Gal (40 μg ml⁻¹) and incubated at 37 °C. Colonies that appeared more blue were selected. Transposon insertion sites were

determined through sequencing the flanking region of the transposon by a semi-random PCR method, as described elsewhere (Levano-Garcia *et al.*, 2005), using random primer #524 and transposon-specific primer #463, followed by the nested primers #525 and #526 (Table 1). β-Galactosidase activities were measured and compared with those of the parental strain (PAO1-P_{*fadBA5*}-*lacZ*) for the three mutants identified as regulatory mutants (Supplementary Fig. S1), where all were grown in LB.

Transduction and complementation of the PAO1-P_{*fadBA5*}-*lacZ*/psrA::Tn-Gm^r mutant. The chromosomal disrupted *psrA*::Tn-Gm^r mutation was transduced back into the original parental PAO1-P_{*fadBA5*}-*lacZ* fusion strain following a protocol described elsewhere

Table 1. Oligonucleotide primers used in this study

Primer name and purpose	Sequence*
Promoter mapping	
#244-SMART-RACE†	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
#279- <i>fadB5</i> -RACE1†	5'-CAACTCACTGAGGGTGAGA-3'
#280- <i>fadB5</i> -RACE2	5'-TTGAACTTGTTGACGGACTC-3'
#281- <i>fadB5</i> -RACE3	5'-GATCGAAATTCAACTCGAC-3'
<i>lacZ</i> fusion construction	
#287- <i>fadAB5</i> (P4)- <i>HindIII</i>	5'-TGAATAAGCTTGCAGCAGAGGGCCTGGAGGAG-3'
#289- <i>fadAB5</i> -P5	5'-GCAAACGCTCGATTTCATACGCC-3'
<i>fadBA5</i> complementation	
#273- <i>fadAB5</i> - <i>EcoRI</i>	5'-CCCGGAATTCCCTTCGACGAACGCTTAG-3'
Sequencing	
#524-HIB17	5'-CGGAATTCGGATNGAYKSNGGNTC-3'
#463-Gm ^R -RT	5'-GAGCAGCCGCGTAGTGAG-3'
#525-3'-Gm-reverse	5'-AAGTACCGCCACCTAAC-3'
#526-marinerTn-reverse	5'-CTGTATGGAACGGGATG-3'
<i>psrA</i> cloning	
#691 <i>psrA</i> -up- <i>NdeI</i>	5'-GAGAACATATGGCCCAGTCGGAAACC-3'
#692 <i>psrA</i> -down- <i>BamHI</i>	5'-GCATGGATCCTGCGATCAGGCCTTGGCG-3'

*Restriction enzyme sites are underlined.

†HPLC-purified and RNase-free oligonucleotides from IDT.

(Choi *et al.*, 2006). Transposon mutants harbouring the *P_{fadB5}-lacZ* reporter gene fusions and four independent isolates of the retransduced PAO1-*psrA*::Tn-Gm^r mutants were grown overnight in PIA + Gm (150 µg ml⁻¹), along with PAO1-*P_{fadBA5}-lacZ* in PIA. Overnight cultures were washed twice and resuspended in an equal volume of LB medium. Resuspended cultures were then diluted 100-fold into fresh LB. Growth curves were performed, and β-galactosidase assays at mid-exponential phase were carried out for each strain.

For the complementation study, the coding region of *psrA* was amplified from PAO1 chromosomal DNA using oligonucleotides #691 and #692. The *NdeI*/*BamHI*-digested PCR product was cloned into pUCP-Nde (Cronin & McIntire, 1999) that had been digested with the same enzymes. Competent cells of the PAO1-*P_{fadBA5}-lacZ*/*psrA*::Tn-Gm^r mutant were prepared and electroporated with 50 ng plasmid DNA of pUCP-Nde-*psrA* or pUCP-Nde, as described elsewhere (Choi *et al.*, 2006), and selected on PIA + carbenicillin (Cb; 500 µg ml⁻¹). Transposon mutants with the *P_{fadBA5}-lacZ* reporter gene fusion, harbouring pUCP-Nde-*psrA* or pUCP-Nde, were grown overnight in PIA + Cb500, washed twice with LB, and diluted 100-fold into fresh LB + Cb500 + 1 % Brij-58 with or without 0.2 % C_{18:1}^{Δ9}. Growth curves were performed and β-galactosidase assays at mid-exponential phase were carried out for each strain.

Purification of His₆-PsrA. His₆-PsrA was expressed and purified using the *E. coli* T7 RNA polymerase expression system. The expression vector pET15b-*psrA* was constructed by cloning the *psrA*-encoding region from pUCP-Nde-*psrA* into pET15b as an *NdeI*-*BamHI* fragment, and then transformed into the BL21-Codonplus(DE3)-RIPL strain (Stratagene). The transformed BL21-Codonplus(DE3)-RIPL strain containing pET15b-*psrA* was grown and PsrA was expressed as described previously (Hoang *et al.*, 2002). After expression, cells were harvested and protein purification was performed as described previously (Hoang *et al.*, 2002). Protein concentration was determined

by Bradford Assay (Bio-Rad), using BSA as a standard, prior to electrophoretic mobility shift assay (EMSA) studies.

EMSA of PsrA binding to *P_{fadBA5}*. Purified His₆-PsrA was incubated with the *P_{fadBA5}* promoter DNA fragment (amplified with oligonucleotides #287 and #289), and a gel mobility shift was performed to demonstrate specific DNA binding. Essentially, 100 ng purified *P_{fadBA5}* DNA fragment was incubated with 1 µg of purified His₆-PsrA in 1 × binding buffer [20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 1 % (w/v) Tween 20, 150 mM KCl] with 1 µg poly(dI-C) and 0.1 µg poly-L-lysine for 30 min at room temperature. For all the FAs used in EMSA (C_{18:1}^{Δ9}, C_{16:0}, C_{14:0}, C_{12:0}, C_{10:0}, C_{8:0}, C_{6:0} and C_{4:0}), 1 mM stocks were made by dissolving the FA in 0.2 % Brij-58 with equimolar KOH. Oleoyl-CoA was diluted to 1 mM with 1 × binding buffer + 0.2 % Brij-58. Master-mix containing binding buffer, poly(dI-C), poly-L-lysine, and the DNA fragment of *P_{fadBA5}* was made for each gel, then FAs (2.5–50 µM) or oleoyl-CoA (50 µM) were added to each aliquot to the appropriate concentration. Purified His₆-PsrA was always the last component to be added to the binding reactions. Each binding reaction was loaded on an 8 % native polyacrylamide gel (pre-run at 90 V for 1 h) and ran for 120 min at 90 V. Gels were stained with SYBR Green as recommended by the manufacturer of EMSA kit E33075 (Invitrogen/Molecular Probes).

RNA isolation, cDNA synthesis and labelling, and Affymetrix GeneChip processing. Starter cultures of PAO1 or the PAO1-*psrA*::Tn-Gm^r mutant derivative were grown in LB for 14 h at 37 °C with aeration, and a 1:100 dilution of the starter cultures was inoculated into 20 ml LB in a 125 ml flask. Cultures were then incubated at 37 °C with shaking and grown up to mid-exponential phase (OD₅₄₀ ~0.6). Cells were then harvested and *P. aeruginosa* mRNA isolated. cDNA syntheses and labelling, as well as GeneChip processing and microarray analysis, were performed as described previously (Son *et al.*, 2007). Analysis was done by conducting pair-wise comparisons

between two replicates of PAO1 and three replicates of PAO1-*psrA::Tn-Gm^r* grown in LB. Genes that exhibited a twofold or greater increase in expression in PAO1-*psrA::Tn-Gm^r* relative to PAO1 form one list (PsrA-repressed genes; Table 2), while genes that showed a twofold or greater increase in expression in PAO1 compared with PAO1-*psrA::Tn-Gm^r* form another (PsrA-activated genes; Table 3).

Gene assignment. Gene assignment was assisted for some genes using COG predictions in the *Pseudomonas* Genome Database (<http://www.pseudomonas.com/index.jsp>).

Real-time TaqMan RT-PCR and data analysis. Primers, probes and real-time RT-PCR were exactly the same as described previously (Son *et al.*, 2007). RNAs used were from the same preparation as described above for microarray experiments. Because normalization by geometric averaging using multiple housekeeping genes has been shown to yield more accurate fold-changes than using a single housekeeping gene (Vandesompele *et al.*, 2002), we opted to use three housekeeping genes for our normalizations. Three housekeeping genes described previously (PA1769, PA1795 and PA1805; Son *et al.*, 2007) were selected and utilized to normalize fold-changes for *lipA* and *fadB5* in the PAO1-*psrA::Tn-Gm^r* mutant compared with PAO1.

RESULTS AND DISCUSSION

FadB5 and FadA5 are involved in LCFA utilization

P. aeruginosa has a large array of β -oxidation enzymes (Stover *et al.*, 2000), and the organism is capable of degrading short-, medium- and long-chain fatty acids (SCFAs, MCFAs and LCFAs) as sole carbon sources. Among FadB and FadA proteins in *P. aeruginosa*, FadB5 (PA3014) and FadA5 (PA3013) have the highest homology to their *E. coli* counterparts (Campbell & Cronan, 2002; Yang *et al.*, 1991), with similarities of 72 % (54 % identity) and 76 % (61 % identity), respectively. Initial characterization demonstrated that the *fadBA5* operon is involved in LCFA utilization (Son *et al.*, 2007). In our previous study, *fadBA5* was not fully characterized with respect to the utilization of FA of different chain lengths as sole carbon sources. In this study, the PAO1- Δ *fadBA5* mutant was further characterized. The PAO1- Δ *fadBA5* mutant had no growth defect when grown on glucose SCFA or MCFA ($C_{4:0}$ to $C_{10:0}$) as sole carbon source, but it showed defects in growth using LCFAs as sole carbon source ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$ and $C_{18:1}^{\Delta 9}$, Fig. 1). The slower growth and lower cell density of the mutant compared with the wild-type suggest a defect in the ability to degrade these LCFAs and the accumulation of growth-inhibitory intermediates. When the *fadBA5* operon was cloned into mini-CTX2 and integrated as a single copy into the PAO1- Δ *fadBA5* mutant background, it completely complemented this mutation (Fig. 1), indicating that there were no other spurious mutations on the chromosome of the mutant strain other than Δ *fadBA5*. There was an incomplete lack of growth using LCFAs as a carbon source for this mutant, because *P. aeruginosa* has two other *fadAB* operons [*fadAB1* (PA1737 and PA1736; Son *et al.*, 2008) and *fadAB4* (PA4786 and PA4785; unpublished data)], which have overlapping functions with *fadBA5* for MCFAs and LCFAs.

Although the functions of *fadAB4* have yet to be fully characterized, we have recently shown that the *fadAB1* operon is induced by MCFAs and to a lesser extent by LCFAs (Son *et al.*, 2008), perhaps for the degradation of these carbon sources, suggesting that FadBA1 has overlapping functions with FadBA5 in the metabolism of different FA chain lengths. We have shown here that *fadBA5* is important for growth on LCFAs *in vitro*. *In vivo* during lung infection, *fadBA5* was significantly induced, suggesting that LCFAs are also available *in vivo* as a source of nutrients (Son *et al.*, 2007). However, the regulation of this operon is undefined and poorly understood in *P. aeruginosa*.

Regulatory region of *fadBA5*

Based on an established non-radioactive method for mapping transcriptional start sites (Tabansky & Nurminsky, 2003), we mapped the promoter of the *fadBA5* operon. Our data shown in Fig. 1 indicate induction of *fadBA5* by palmitate ($C_{16:0}$). Therefore, to map the *fadBA5* transcript start site, we utilized RNA isolated from PAO1 grown in $1 \times$ M9 minimal media containing palmitic acid as the sole carbon source to induce the expression of these *fad* genes. We mapped the transcript start site for *fadBA5* to 185 bp upstream of the start codon of *fadB5* (Fig. 2a, c). We utilized free software (http://nostradamus.cs.rhul.ac.uk/~leo/sak_demo/) for the prediction of prokaryotic σ^{70} promoters using sequence alignment kernel methods (Gordon *et al.*, 2003), which predicted a perfect match to our *fadBA5* transcriptional start-site mapping data. This gives further confirmation of the promoter mapping method. There is a pseudo-palindromic sequence, with one base mismatch, at the -10 and -35 region of this mapped σ^{70} promoter (blue arrows in Fig. 2a). Since the mapping method is based on RT-PCR and is semiquantitative, the band in Fig. 2(b) (lane 1) indicated the expression of *fadBA5* in the exponential phase during active degradation of palmitate as the sole carbon source, and the expression of *fadBA5* was not detected in early or late stationary phase (Fig. 2b).

LCFAs induce *fadBA5*

Since we had shown that the *fadBA5* operon is required for growth on LCFAs (Fig. 1) and we had mapped the promoter of *fadBA5* (Fig. 2), we next wanted to determine which FA chain lengths induce *fadBA5*. A P_{fadBA5} -*lacZ* fusion was constructed in mini-CTX2 and introduced as a stable single copy into the *P. aeruginosa* chromosome to study the regulation of *fadBA5* by LCFAs. We analysed the expression of the P_{fadBA5} -*lacZ* fusion in strain PAO1 grown in $1 \times$ M9 glucose media $\pm$ FA of different chain lengths. As shown in Fig. 3, *fadBA5* was tightly repressed when grown with glucose as the sole carbon source at mid-exponential phase. This operon was also repressed in the presence of FA from $C_{4:0}$ to $C_{10:0}$ at mid-exponential phase. However, at mid-exponential phase, *fadBA5* was

Table 2. Genes repressed by PsrA as determined by microarray

Genes proposed to contribute to FA metabolism are in bold type.

PA number	Gene	Description	Fold-change*
PA0013	<i>yhhQ</i>	Conserved hypothetical protein	2.1
PA0040		Conserved hypothetical protein	2.3
PA0150		Probable transmembrane sensor	2.2
PA0164		Probable gamma-glutamyltranspeptidase	2.3
PA0263	<i>hcpC</i>	Secreted protein Hcp	2.3
PA0349		Hypothetical protein	2.2
PA0506	<i>fadE</i>	Probable acyl-CoA dehydrogenase	13.7
PA0726		Hypothetical protein of bacteriophage Pfl	2.1
PA0782	<i>putA</i>	Proline dehydrogenase PutA	6.8
PA0783	<i>putP</i>	Sodium/proline symporter PutP	4.9
PA1511		Conserved hypothetical protein	2.1
PA1657		Conserved hypothetical protein	2.4
PA1658		Conserved hypothetical protein	2.2
PA1659		Hypothetical protein	2.3
PA1660		Hypothetical protein	2.2
PA1661		Hypothetical protein	2.2
PA1662		Probable ClpA/B-type protease	2.2
PA1663		Probable transcriptional regulator	2.5
PA1665		Hypothetical protein	2.9
PA1666		Hypothetical protein	2.2
PA1667		Hypothetical protein	2.6
PA1668		Hypothetical protein	3.0
PA1669		Hypothetical protein	3.1
PA1670	<i>stp1</i>	Serine/threonine phosphoprotein phosphatase Stp1	2.6
PA1827		Probable short-chain dehydrogenase	2.1
PA1829		Hypothetical protein	3.1
PA1830		Hypothetical protein	5.1
PA2404		Hypothetical protein	2.4
PA2462		Hypothetical protein	2.2
PA2671	<i>hplX</i>	Hypothetical protein	2.7
PA2845		Hypothetical protein	2.1
PA2862	<i>lipA</i>	Lactonizing lipase precursor	2.0 (2.2)
PA2951	<i>etfA</i>	Electron transfer flavoprotein alpha-subunit	2.1
PA2952	<i>etfB</i>	Electron transfer flavoprotein beta-subunit	2.4
PA2953	<i>fixC</i>	Electron transfer flavoprotein, ubiquinone oxidoreductase	8.0
PA3006	<i>psrA</i>	Transcriptional regulator PsrA	6.2
PA3013	<i>fadA5</i>	Fatty-acid oxidation complex beta-subunit	8.3
PA3014	<i>fadB5</i>	Fatty-acid oxidation complex alpha-subunit	9.7 (14.5)
PA3059	<i>pelF</i>	Hypothetical protein	2.2
PA3327		Probable non-ribosomal peptide synthetase	2.1
PA3329		Hypothetical protein	2.1
PA3331	<i>cypX</i>	Cytochrome P450	2.4
PA3332		Conserved hypothetical protein	2.2
PA3334		Probable acyl carrier protein	2.0
PA3335		Hypothetical protein	2.5
PA3381	<i>phnF</i>	Probable transcriptional regulator	2.2
PA3487	<i>pldA</i>	Phospholipase D	2.2
PA3874	<i>narH</i>	Respiratory nitrate reductase beta chain	2.4
PA3875	<i>narG</i>	Respiratory nitrate reductase alpha chain	3.3
PA3876	<i>narK2</i>	Nitrite extrusion protein 2	2.9
PA4773		Hypothetical protein	2.8
PA4774		Hypothetical protein	2.1
PA4881		Hypothetical protein	2.1
PA5266		Conserved hypothetical protein	2.3

*Indicated are average fold-changes for six pair-wise comparisons (with P values ≤ 0.05) for the PAO1-*psrA*::Tn-Gm^r mutant versus wild-type PAO1. The fold-changes determined by real-time TaqMan RT-PCR are in parentheses.

Fig. 2. SMART mapping of the transcript start site for the *fadBA5* operon. (a) *fadBA5* coding sequences and the predicted -10 and -35 promoter regions (boxed) are shown relative to FadB5 and FadA5 start codons. The FadB5 stop codon is separated by only 30 bp from the start of FadA5. Three black arrows indicate primers 1, 2 and 3 (#279, #280 and #281) used for mapping. There is a dyad symmetrical sequence (blue arrows) just upstream of this transcriptional start site (bold and underlined letter **A**). Underlining in green (number 1) and red overlining (number 2) indicate two possible *PsrA* binding sites. (b) Only one SMART product was observed after PCR amplification of the cDNA with SMART primer and primer 2 (oligonucleotides #244 and #280); lane 1, mRNA isolated from exponential phase of PAO1 grown on palmitic acid ($C_{16:0}$); lane 2, mRNA isolated from the same culture in early stationary phase; lane 3, mRNA isolated from late-stationary phase. We used the $1\times M9$ +palmitic acid growth condition to induce the transcription of the *fadBA5* operon. (c) Sequencing of the ACCCA band of panel (b) with primer 3 (oligonucleotide #281). Displayed are the reverse-complement sequences, showing the *fadBA5* transcription start site (indicated by an arrow) and the underlined SMART primer sequences.

induced by LCFAs ($C_{14:0}$, $C_{16:0}$ and $C_{18:1}^{\Delta 9}$) and to a lesser extent by $C_{12:0}$ (Fig. 3). We had determined that at 40 mM glucose in all media with and without FA, the growth rates for the fusion strain were identical throughout the exponential phase for all cultures (data not shown). Therefore, the induction of the *fadBA5* operon by LCFAs at mid-exponential phase was not due to differences in growth rates. This induction of *fadBA5* by LCFAs (Fig. 3) also correlates well with its metabolic function and with its growth on similar FA chain lengths (Fig. 1).

Identification of *PsrA* (PA3006) as the *fadBA5* regulator

To initiate studies on the *fadBA5* regulation mechanism, we searched for a regulator of *fadBA5*. We utilized a transposon mutagenesis approach similar to that described elsewhere, using the gentamicin-resistant (Gm^r) transposon vector pBT20 (Kulasekara *et al.*, 2005) to randomly mutagenize the chromosome of strain PAO1 carrying a

P_{fadBA5}-lacZ fusion. Since it is our hypothesis that there is a repressor that regulates *fadBA5*, we looked for Gm^r -transposon mutants of PAO1-*P_{fadBA5}-lacZ* that were bluer on X-Gal-containing media, signifying transposon inactivation of a potential negative regulator. We sequenced 67 bluer transposon mutants as described elsewhere (Levano-Garcia *et al.*, 2005) (Supplementary Table S1) to identify the inactivation of any potential regulator, and this is summarized in Fig. 4. It is interesting to see that a large number of bluer colonies affecting *fadBA5* expression were associated with LPS biosynthesis (*wpb* genes), suggesting a possible connection between Fad and LPS biosynthesis. However, further research is required to determine the connection between Fad and LPS biosynthesis. Of interest were three probable transcriptional regulators (PA2601, PA3006 or *PsrA*, and PA3508; Supplementary Table S1). More thorough measurements of β -galactosidase activities of these three regulatory mutants showed increased β -galactosidase activities (> 2 -fold) for the PA2601::Tn- Gm^r and *psrA*::Tn- Gm^r mutants, which significantly

Fig. 3. Expression of the *fadBA5* operon during the mid-exponential growth phase in 1× M9+40 mM glucose with or without 0.1 % FA (C_{4:0}, C_{6:0}, C_{8:0}, C_{10:0}, C_{12:0}, C_{14:0}, C_{16:0} or C_{18:1}^{Δ9}). Growth with glucose alone or glucose with C_{4:0} to C_{10:0} did not induce *fadBA5*, whereas growth on glucose plus LCFA C_{12:0} to C_{18:1}^{Δ9} significantly induced *fadBA5*. Growth rates of strain PAO1-*P_{fadBA5}-lacZ* in different media, with and without FA, are exactly identical (data not shown). β-Galactosidase activities are mean ± SEM of triplicates.

affected the expression of *fadBA5* during exponential phase (Supplementary Fig. S1). Southern hybridization analysis indicated that there were two transposons in strain PAO1-*P_{fadBA5}-lacZ*/PA2601::Tn-Gm^r and a single transposon in the chromosome of strain PAO1-*P_{fadBA5}-lacZ*/*psrA*::Tn-Gm^r (Supplementary Fig. S1). We separated and sequenced the other mutation in the strain carrying the PA2601::Tn-Gm^r mutation, and this indicated that there was another

transposon mutation in the LPS biosynthetic gene, *wbpM*. Separating these two mutations by retransduction of chromosomal fragments (Choi *et al.*, 2006) into the PAO1-*P_{fadBA5}-lacZ* fusion strain indicated that PA2601 does not affect the expression of *fadBA5* (data not shown), and that the increased *P_{fadBA5}* promoter activity was due to the *wbpM* mutation. In addition, purified His-tagged PA2601 did not bind to the *fadBA5* promoter region (data not shown), showing further that PA2601 was not involved in the regulation of *fadBA5*.

Constitutive expression of *fadBA5* was due to a single *psrA*::Tn-Gm^r mutation (Fig. 5 and Supplementary Fig. S1). To rule out potential *fadBA5* promoter mutation(s) causing constitutive *P_{fadBA5}-lacZ* expression in the *psrA*::Tn-Gm^r mutant, we transduced this mutation back into the parental PAO1-*P_{fadBA5}-lacZ* fusion strain, as described elsewhere (Choi *et al.*, 2006). Four independent transductants were again measured for the expression of the *P_{fadBA5}-lacZ* fusion, and all four mutants showed constitutive expression of *fadBA5* (Fig. 5a), indicating that the derepression of *fadBA5* is due to a single mutation in *psrA*. We further cloned and complemented the *psrA*::Tn-Gm^r mutation, and the clones complemented the *psrA* mutation to repress *P_{fadBA5}-lacZ* back to wild-type levels (Fig. 5b). In addition, upon adding oleate (C_{18:1}^{Δ9}) to the LB media, these complemented clones derepressed *fadBA5* similarly to wild-type. The vector-alone control showed no repression of *fadBA5* (Fig. 5b), and no further increase in *fadBA5* expression was observed upon addition of the FA oleate to the vector-alone control strain. Together, these data for the PAO1-*psrA*::Tn-Gm^r mutant indicate a requirement for LCFA to induce the *fadBA5* operon in LB media. The growth phase-independent constitutive expression of *fadBA5* in the PAO1-*P_{fadBA5}-lacZ*/*psrA*::Tn-Gm^r strain suggests a direct regulatory role of PsrA (PA3006) on *fadBA5* (Supplementary Fig. S1a). We

Fig. 4. Transposon insertion sites grouped according to protein functions. The functional groups were assigned based on the *Pseudomonas* Genome Database. Overall, 10% of the transposon insertion sites are in three probable transcriptional regulators (PA2601, PA3006 and PA3508), shown in Supplementary Table S1. A significant proportion were observed to occur in LPS-related genes.

Fig. 5. (a) The disrupted PA3006 (*PsrA*) chromosomal mutation was transduced back into the original parental PAO1-*P_{fadBA5}-lacZ* fusion strain. All four retransduced mutants showed deregulation of the *P_{fadBA5}-lacZ* fusion similar to the original *psrA*::Tn-Gm^r mutant, confirming that the derepression of *fadBA5* is due to a single mutation in *psrA*. (b) Complementation of the *psrA* mutation, in which the coding region of *psrA* from the initiation codon to the stop codon was cloned into pUCP-Nde and driven by the *lac* promoter. The pUCP-PA3006 and the pUCP-Nde vector control were transformed into the PAO1-*psrA*::Tn-Gm^r mutant carrying the chromosomal *P_{fadBA5}-lacZ* fusion. When grown in LB, the vector-alone control showed no repression of the *P_{fadBA5}-lacZ* fusion in the PAO1-*psrA*::Tn-Gm^r mutant, whereas three independent pUCP-PA3006 (pUCP-Nde-*psrA*) clones showed complementation and repression of this fusion back to wild-type level in LB. As expected, this repression was also relieved by adding FA (oleate, C_{18:1} ^{Δ 9}) to the LB media. Strains carrying the pUCP control plasmid (in LB and LB + C_{18:1} ^{Δ 9}) show that the addition of oleate alone cannot further induce the *fadBA5* operon, suggesting that only one pathway, through *PsrA*, exists to regulate *fadBA5*. For both (a) and (b), all cultures were grown to mid-exponential phase for β -galactosidase measurements and all cultures had identical growth rates in LB and LB + C_{18:1} ^{Δ 9}.

demonstrate below that *PsrA* binds directly to the *fadBA5* promoter region.

***PsrA* binds to the promoter region of *fadBA5* and is inhibited by LCFAs**

To demonstrate direct binding of purified His₆-*PsrA* to the promoter region of *fadBA5* (*P_{fadBA5}*), we performed *in vitro* EMSA. The data showed that *PsrA* binds specifically to *P_{fadBA5}* (Fig. 6a, lanes 3 and 4) and that His₆-*PsrA* does not bind to other tested promoters or DNA (e.g. the ~400 bp *aacC1* gentamicin-resistance promoter; data not shown). Since *PsrA* regulates *fadBA5* and *fadBA5* is involved in growth on LCFAs, we wanted to see if free LCFAs or long-chain acyl-CoAs could inhibit the binding of *PsrA* to *P_{fadBA5}* by acting as an inducer. The LCFA oleate could inhibit *PsrA* binding. However, oleoyl-CoA, which acts as an inducer of *E. coli fad* genes by binding to FadR (DiRusso *et al.*, 1992), could not inhibit *PsrA* binding to *P_{fadBA5}* (Fig. 6a, b). We next tested the range of FA chain lengths that *PsrA* could recognize and thereby inhibit the shifting of *P_{fadBA5}*. Only LCFAs (C_{12:0}, C_{14:0}, C_{16:0} and C_{18:1} ^{Δ 9}), but not MCFAs or SCFAs, could visibly inhibit the binding of

PsrA to *P_{fadBA5}* *in vitro* (Fig. 6c). The inhibition of *PsrA* binding to *P_{fadBA5}* DNA was in a gradient fashion from C_{12:0} to C_{14:0} and from C_{16:0} to C_{18:1} ^{Δ 9} (Fig. 6c), which is consistent with the induction of *P_{fadBA5}-lacZ* by LCFAs (Fig. 3) and the growth characteristics of the Δ *fadBA5* mutant (Fig. 1). Together, these data, as well as the growth curves and gene fusion experiments (Figs 1 and 3), consistently indicate that LCFAs are the inducer of *fadBA5*, and bind to *PsrA* to derepress the *fadBA5* operon in *P. aeruginosa*.

The protein:DNA and inducer:protein ratios used here are comparable to those of an earlier study (DiRusso *et al.*, 1992). Since we utilized SYBR Green from an established EMSA kit (E33075, Invitrogen/Molecular Probes) as an alternative to radioactivity for this DNA binding study, a larger amount of DNA (100 ng; ~1.28 pmol) was used to visualize the *P_{fadBA5}* DNA band rather than that typically used for radioactivity-based EMSA (25 attomol DNA) (DiRusso *et al.*, 1992). Therefore, the protein (1 μ g His₆-*PsrA*) to DNA mole ratio was approximately 30:1 in our study. This ratio is significantly lower than that in the earlier radioactivity-based study with the *E. coli* FadR

Table 3. Genes activated by PsrA as determined by microarray

PA number	Gene	Description	Fold-change*
PA0039		Hypothetical protein	2.3
PA0059	<i>osmC</i>	Osmotically inducible protein OsmC	2.8
PA0106	<i>coxA</i>	Cytochrome <i>c</i> oxidase, subunit I	2.2
PA0107		Conserved hypothetical protein	2.2
PA0122		Conserved hypothetical protein	2.1
PA0131		Hypothetical protein	2.4
PA0132	<i>oapT</i>	Beta-alanine-pyruvate transaminase	2.1
PA0180		Probable chemotaxis transducer	2.4
PA0265	<i>gabD</i>	Succinate-semialdehyde dehydrogenase	2.1
PA0266	<i>gabT</i>	4-Aminobutyrate aminotransferase	2.4
PA0296		Probable glutamine synthetase	2.1
PA0355	<i>pfpI</i>	Protease PfpI	2.3
PA0484		Conserved hypothetical protein	2.2
PA0567	<i>yqaE</i>	Conserved hypothetical protein	2.3
PA0704		Probable amidase	2.4
PA0784		Probable transcriptional regulator	2.2
PA1041		Probable outer-membrane protein precursor	3.0
PA1048		Probable outer-membrane protein precursor	2.5
PA1172	<i>napC</i>	Cytochrome <i>c</i> -type protein NapC	2.5
PA1173	<i>napB</i>	Cytochrome <i>c</i> -type protein NapB precursor	2.1
PA1174	<i>napA</i>	Periplasmic nitrate reductase protein NapA	2.3
PA1176	<i>napF</i>	Ferredoxin protein NapF	2.4
PA1214		Hypothetical protein	2.1
PA1215		Hypothetical protein	2.6
PA1216		Hypothetical protein	2.8
PA1217		Probable 2-isopropylmalate synthase	2.6
PA1218		Hypothetical protein	3.0
PA1221		Hypothetical protein	2.0
PA1289		Hypothetical protein	2.8
PA1323		Hypothetical protein	3.5
PA1324		Hypothetical protein	3.8
PA1440		Hypothetical protein	2.5
PA1562	<i>acnA</i>	Aconitate hydratase 1	2.0
PA1745		Hypothetical protein	2.1
PA1784		Hypothetical protein	3.7
PA1874		Hypothetical protein	10.6
PA1875	<i>opmL</i>	Probable outer-membrane protein precursor	13.3
PA1876		Probable ATP-binding/permease fusion ABC transporter	6.0
PA1881		Probable oxidoreductase	2.5
PA1888		Hypothetical protein	2.1
PA1914	<i>hvn</i>	Conserved hypothetical protein	11.9
PA2030		Hypothetical protein	2.2
PA2031		Hypothetical protein	2.0
PA2071	<i>fusA2</i>	Elongation factor G	2.3
PA2134		Hypothetical protein	2.3
PA2146	<i>yciG</i>	Conserved hypothetical protein	2.3
PA2152		Probable trehalose synthase	2.1
PA2159		Conserved hypothetical protein	3.5
PA2160	<i>glgX</i>	Probable glycosyl hydrolase	2.6
PA2164		Probable glycosyl hydrolase	2.3
PA2167		Hypothetical protein	2.6
PA2169		Hypothetical protein	2.4
PA2170		Hypothetical protein	2.2
PA2171		Hypothetical protein	3.7
PA2172		Hypothetical protein	3.7
PA2173		Hypothetical protein	3.7
PA2176		Hypothetical protein	2.1
PA2231	<i>pslA</i>	Probable glycosyl transferase	2.1
PA2232	<i>pslB</i>	Probable phosphomannose isomerase/GDP-mannose pyrophosphorylase	2.2

Table 3. cont.

PA number	Gene	Description	Fold-change*
PA2233	<i>pslC</i>	Probable glycosyl transferase	2.1
PA2234	<i>pslD</i>	Probable exopolysaccharide transporter	2.1
PA2235	<i>pslE</i>	Hypothetical protein	2.1
PA2236	<i>pslF</i>	Hypothetical protein	2.3
PA2290	<i>gcd</i>	Glucose dehydrogenase	2.3
PA2368		Hypothetical protein	2.1
PA2378		Probable aldehyde dehydrogenase	2.5
PA2433		Hypothetical protein	3.6
PA2442	<i>gcvT2</i>	Glycine cleavage system protein T2	2.1
PA2444	<i>glyA2</i>	Serine hydroxymethyltransferase	2.1
PA2445	<i>gcvP2</i>	Glycine cleavage system protein P2	2.2
PA2446	<i>gcvH2</i>	Glycine cleavage system protein H2	2.6
PA2566		Conserved hypothetical protein	2.8
PA2573		Probable chemotaxis transducer	2.1
PA2717	<i>cpo</i>	Chloroperoxidase precursor	2.4
PA2721		Hypothetical protein	2.0
PA2754		Conserved hypothetical protein	2.0
PA2771		Conserved hypothetical protein	2.2
PA2779		Hypothetical protein	2.2
PA2815	<i>yafH</i>	Probable acyl-CoA dehydrogenase	2.1
PA2937		Hypothetical protein	2.0
PA2939	<i>pepB</i>	Probable aminopeptidase	3.5
PA3032	<i>snr1</i>	Cytochrome c Snr1	2.1
PA3041	<i>yqjE</i>	Hypothetical protein	2.4
PA3182	<i>pgl</i>	6-Phosphogluconolactonase	2.9
PA3195	<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	2.6
PA3250		Hypothetical protein	2.1
PA3340		Hypothetical protein	2.4
PA3347		Hypothetical protein	2.3
PA3370		Hypothetical protein	2.1
PA3416		Probable pyruvate dehydrogenase E1 component, beta chain	2.0
PA3418	<i>ldh</i>	Leucine dehydrogenase	2.0
PA3688		Hypothetical protein	2.2
PA3691		Hypothetical protein	3.3
PA3692		Probable outer-membrane protein precursor	2.7
PA3723	<i>yqjM</i>	Probable FMN oxidoreductase	2.4
PA3819	<i>yefJ</i>	Conserved hypothetical protein	2.4
PA3888		Probable permease of ABC transporter	2.3
PA3891		Probable ATP-binding component of ABC transporter	2.4
PA3929	<i>cioB</i>	Cyanide insensitive terminal oxidase	2.1
PA4015		Conserved hypothetical protein	2.2
PA4078		Probable nonribosomal peptide synthetase	3.3
PA4175	<i>prpL</i>	Pvds-regulated endoprotease, lysyl class	2.7
PA4209	<i>phzM</i>	Probable phenazine-specific methyltransferase	2.7
PA4293	<i>pprA</i>	Two-component sensor PprA	3.9
PA4294		Hypothetical protein	3.7
PA4296	<i>pprB</i>	Two-component response regulator PprB	2.6
PA4297	<i>tadG</i>	TadG (COG predictions: predicted membrane protein)	2.4
PA4298		Hypothetical protein	7.0
PA4299	<i>tadD</i>	TadD (COG predictions: Flp pilus assembly protein)	5.1
PA4300	<i>tadC</i>	TadC (COG predictions: Flp pilus assembly protein)	3.5
PA4301	<i>tadB</i>	TadB (COG predictions: Flp pilus assembly protein)	2.6
PA4302	<i>tadA</i>	TadA ATPase (COG predictions: Flp pilus assembly protein, ATPase CpaF)	3.7
PA4304	<i>rcpA</i>	RcpA (COG predictions: Flp pilus assembly protein, secretin CpaC)	3.5
PA4305	<i>rcpC</i>	RcpC (COG predictions: Flp pilus assembly protein, secretin CpaB)	3.5
PA4306	<i>flp</i>	Type IVb pilin, Flp	7.4
PA4311		Conserved hypothetical protein	2.6
PA4362		Hypothetical protein	2.1
PA4495		Hypothetical protein (COG predictions: periplasmic/secreted protein)	2.1
PA4607	<i>mscL</i>	Hypothetical protein	2.1

Table 3. cont.

PA number	Gene	Description	Fold-change*
PA4614		Conductance mechanosensitive channel	2.1
PA4638		Hypothetical protein	2.2
PA4648		Hypothetical protein	9.0
PA4649		Hypothetical protein	5.1
PA4650		Hypothetical protein	3.3
PA4651		Probable pili assembly chaperone	8.2
PA4652		Hypothetical protein	2.7
PA4653		Hypothetical protein	4.3
PA4733	<i>acsB</i>	Acetyl-coenzyme A synthetase	2.3
PA4738	<i>yjbJ</i>	Conserved hypothetical protein	2.9
PA4739		Conserved hypothetical protein	2.8
PA4876	<i>osmE</i>	Osmotically inducible lipoprotein OsmE	3.7
PA4880		Probable bacterioferritin	3.5
PA4915		Probable chemotaxis transducer	2.1
PA4925		Conserved hypothetical protein	2.4
PA5058	<i>phaC2</i>	Poly(3-hydroxyalkanoic acid) synthase 2	2.3
PA5212		Hypothetical protein	2.2
PA5312	<i>kauB</i>	Probable aldehyde dehydrogenase	2.3
PA5313	<i>paaT</i>	Probable pyridoxal-dependent aminotransferase	3.0
PA5481		Hypothetical protein	2.5
PA5482		Hypothetical protein	2.3
PA5528		Hypothetical protein	2.2
PA5546		Conserved hypothetical protein	2.1

*Indicated are average fold-changes for six pair-wise comparisons (with *P* values ≤ 0.05) for the wild-type PAO1 versus the PAO1-*psrA*::Tn-Gm^r mutant.

(DiRusso *et al.*, 1992), where the protein:DNA mole ratio used was 1000:1. Along with the inability of His₆-PsrA to bind other control DNA (data not shown), this appropriate protein:DNA ratio further supports the hypothesis that the His₆-PsrA binding to *P_{fadBA5}* DNA observed in our study is specific. Given the high concentrations of DNA and His₆-PsrA used, the 50 μ M FA concentration used in this study is reasonable, yielding an inducer (FA) to protein mole ratio of 27:1. This value is comparable to that in the earlier *E. coli* FadR study (DiRusso *et al.*, 1992), which utilized an inducer (acyl-CoA) to protein mole ratio of 50:1. Our data strongly indicate that the binding of His₆-PsrA to *P_{fadBA5}* DNA is specific and is inhibited by LCFAs. The data support the suggestion that the novel signals for PsrA to regulate *fadBA5* are LCFAs.

PsrA affects gene expression globally

It has been suggested elsewhere that PsrA, a member of the TetR family of repressors, could affect global gene expression (Kojic *et al.*, 2005; Shen *et al.*, 2006), including activation of *rpoS* (Kojic *et al.*, 2002) and the type III secretion system *exsCEBA* operon (Shen *et al.*, 2006). A proteomic study has shown that PsrA influences the expression of a few proteins, including FadBA5, but the signal (inducer) and mechanism of regulation by PsrA have not been demonstrated (Kojic *et al.*, 2005). The full array of genes influenced by PsrA has not been shown. Hence, we

wanted to utilize a genomic approach to determine the extent of PsrA-regulated genes, using Affymetrix GeneChips to show differential expression between wild-type PAO1 and the PAO1-*psrA*::Tn-Gm^r mutant. We grew both strains, with identical growth rates in LB (data not shown), and harvested both strains at the same cell density at mid-exponential phase to determine the difference in gene expression. Table 2 lists genes negatively affected by PsrA. First, PsrA regulates itself, which has been demonstrated elsewhere (Kojic *et al.*, 2002), and our genomic data indicate a difference of 6.2-fold at mid-exponential phase. Recently, the expression of *psrA* and *fadBA5* *in vivo* in the CF lung have also been observed (Son *et al.*, 2007), suggesting that the bacteria sense free FA in the lung for PsrA to derepress itself and *fadBA5*. Second, several genes involved in FA metabolism were also negatively regulated by PsrA (Table 2), including *fadBA5* (PA3014 and PA3013) and another possible *fad* gene, *fadE* (PA0506). Electron transport genes were significantly induced (PA2951–2953), along with cytochrome P450 (PA3331) (Table 2). This group of genes is significant for Fad, because the result of β -oxidation is the production of reducing power in the form of FADH₂ and NADH. Hence, Fad is closely coupled to electron transport. It has been suggested that the *E. coli* FadE contains sequences that bind and shuffle its electrons (FADH₂) to electron transport protein(s) (Campbell & Cronan, 2002). Cytochrome P450 has been demonstrated to be involved in the oxidation of

Fig. 6. EMSA indicates that direct binding of His₆-PsrA to the *fadBA5* promoter region (*P_{fadBA5}*) is specifically inhibited by LCFAs. (a) Binding of PsrA to *P_{fadBA5}* is inhibited by increasing concentrations of oleate (2.5, 5, 10, 25 and 50 μ M). (b) Binding of PsrA to *P_{fadBA5}* is not inhibited by increasing concentration of oleoyl-CoA (2.5, 5, 10, 25 and 50 μ M). (c) Binding of PsrA to *P_{fadBA5}* is inhibited by 50 μ M of each LCFA (*C_{12:0}*, *C_{14:0}*, *C_{16:0}* and *C_{18:1}^{Δ9}*), and inhibition by other MCFAs and SCFAs was not detected at this concentration. The amount of *P_{fadBA5}* DNA used was 100 ng, and the amount of His₆-PsrA used in each reaction was 1 μ g. To help solubilize FAs, especially LCFAs, Brij-58 was added at 0.01 % to all tubes. Lanes 3 and 4 for each gel are without and with 0.01 % Brij-58, respectively; as observed, there was no effect on the binding of PsrA to *P_{fadBA5}* DNA in the presence of this non-ionic detergent. All pictures are negative images of polyacrylamide gels stained with SYBR Green.

LCFAs (Salaun & Helvig, 1995), and it exists within the gene cluster for FA catabolism of *Pseudonocardia autotrophica* that is also regulated by LCFAs (Chen *et al.*, 2005). It is interesting that we saw a phospholipase D (PA3487) and a lipase (*lipA*, PA2862) also negatively regulated by PsrA. Of particular significance is the lipase, which could potentially liberate LCFAs from phospholipids (e.g. lung surfactant phosphatidylcholine) (Son *et al.*, 2007). These LCFAs would then feed directly into β -oxidation through *fadBA5*. The microarray data were confirmed and were in close agreement with real-time RT-PCR data for both *fadB5* and *lipA* (Table 2 in parentheses), supporting the role of PsrA as a repressor of genes in the Fad pathway. The PsrA binding or recognition consensus sequence has been demonstrated to be G/CAAACN₂₋₄GTTTG/C (Kojic *et al.*,

2005; Shen *et al.*, 2006). Out of hundreds of genes regulated by PsrA in the microarray data (Tables 2 and 3), we focused on genes that potentially contribute to Fad. We found and aligned the consensus sequences upstream of these genes involved in Fad (Fig. 7), and this suggested a direct regulation mechanism of these genes by PsrA. It is interesting that there are two potential PsrA binding consensus sequences in the *fadBA5* promoter region (Figs 2a and 7). The location of these binding sites relative to the -10 and -35 sequences confirms the role of PsrA as a repressor for the *fadBA5* operon (Fig. 2a). Together with the microarray data and the existence of PsrA binding consensus sequences upstream, several potential *fad* genes seem to be directly repressed by PsrA, suggesting that one of its major physiological roles is as a regulator of FA metabolism.

<i>fadE</i>	(PA0506)	-136	<u>CAAACGCCTGTTTG</u>	-123
<i>lipA</i>	(PA2862)	-218	<u>CAAACGAGCGTTTC</u>	-205
<i>etfBA</i>	(PA2951-2)	-216	<u>CAAACAAACGTTTG</u>	-202
<i>fixC</i>	(PA2953)	-119	<u>CAAACGTTTGTTTG</u>	-106
<i>psrA</i>	(PA3006)	-29	<u>GAAACGTATGTTTC</u>	-16
<i>fadBA5-1</i>	(PA3013-4)	-190	<u>CAAACGGGCGTATG</u>	-177
<i>fadBA5-2</i>	(PA3013-4)	-176	<u>GAATCGAGCGTTTG</u>	-163

Fig. 7. Alignment of the PsrA-binding consensus sequences upstream of genes potentially contributing to Fad. The consensus sequences of the PsrA binding motif (G/CAAACN₂₋₄GTTTG/C) are underlined. Numbers indicate the relative position of the nucleotide from the start codon for each gene.

Conversely, PsrA positively activates several genes. Table 3 shows the list of genes potentially activated by PsrA, directly or indirectly. The majority of the genes regulated by PsrA are unknown hypothetical proteins, the future characterization of which will be interesting. One *fadE* homologue, PA2815, seems to be positively regulated by PsrA. Several genes involved in the production of type IV pili were expressed, and seem to be activated by PsrA. It is curious that the stationary-phase sigma factors *rpoS* and *exsC* of the type III secretion system, shown elsewhere to be positively regulated by PsrA (Kojic *et al.*, 2002; Shen *et al.*, 2006), were not present in Table 3. However, our GeneChip data are only representative of a single point during exponential growth, and the regulation of these two genes could occur later in the growth phases or be less than the twofold cut-off for our growth conditions.

Conclusions

Our laboratory has been interested in Fad of *P. aeruginosa* with respect to the lung environment (Son *et al.*, 2007). We have previously demonstrated the expression of genes *in vivo* in the lungs of CF patients, including *fadBA5*, which is required for PC and FA degradation *in vitro* (Son *et al.*, 2007). Cleavage of PC by *P. aeruginosa* phospholipases and subsequently by lipases (Martinez *et al.*, 1999; Rosenau & Jaeger, 2000) will free the readily available LCFAs to the bacteria. These FAs are made mostly of C_{16:0} (50–60 %) and ~10–20 % of each of C_{14:0}, C_{16:1}, C_{18:1} and C_{18:2} (Postle *et al.*, 1999). These LCFAs would serve as inducers of Fad enzymes to metabolize LCFAs via β -oxidation. Indeed, the expression of *psrA* and *fadBA5* has also been observed *in vivo* in the CF lung (Son *et al.*, 2007), suggesting that the bacteria sense free FAs in the lung for PsrA to derepress itself and *fadBA5* to metabolize LCFAs.

We initiated this study to decipher the regulatory mechanism of the *P. aeruginosa fadBA5* operon. *fadBA5* was shown to be important for the utilization of LCFAs as sole carbon sources (Fig. 1). We mapped the transcript start site and identified the promoter region of *fadBA5* (Fig. 2). The promoter region of *fadBA5* was utilized to construct a P_{*fadBA5*}-*lacZ* chromosomal fusion, allowing us

to demonstrate the regulation of *fadBA5* by LCFAs in *P. aeruginosa* (Fig. 3). The PAO1-P_{*fadBA5*}-*lacZ* fusion strain was used in transposon mutagenesis to identify a regulator for *fadBA5*, which is PsrA (PA3006). The DNA recognition sequence for PsrA binding is well established (Kojic *et al.*, 2005; Shen *et al.*, 2006). Downstream of the *fadBA5* promoter region, there were two putative PsrA binding sites (Figs 2a and 7). Our EMSA confirmed this, and indicated that PsrA binds to P_{*fadBA5*} *in vitro* and that this binding is inhibited by LCFAs and not by long-chain acyl-CoAs (Fig. 6). FadR, which regulates *fad* genes in *E. coli*, has been shown to bind to acyl-CoAs of $\geq$ C12 to derepress Fad (DiRusso *et al.*, 1992; Henry & Cronan, 1992). This represents an interesting and novel difference between *P. aeruginosa* and *E. coli* LCFA degradation. Whether free FAs get into the cytoplasm to bind PsrA, or whether PsrA interacts closely with the membrane or other membrane proteins to obtain free FAs from the membrane, is a current enigma. Thus, how PsrA gains access to free FAs will be an interesting subject for future investigations. We further showed that PsrA is a global regulator of several genes, both positively and negatively. Among the genes repressed by PsrA were the ones leading to LCFA degradation, including *fadBA5* and *lipA*, and this was confirmed by real-time RT-PCR. Finally, although PsrA seems to regulate some virulence genes (*lipA* and type IV pili genes), we focused on *fadBA5* and propose here that PsrA is a β -oxidation regulator responding to LCFAs. It is exciting to anticipate the full physiological role of PsrA, once the functions of the many hypothetical proteins that it regulates are determined.

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