Functional genomics of PycR, a LysR family transcriptional regulator essential for maintenance of *Pseudomonas aeruginosa* in the rat lung

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The human opportunistic pathogen *Pseudomonas aeruginosa* is the major cause of morbidity and mortality of cystic fibrosis patients and is responsible for a variety of infections in compromised hosts. Using PCR-based signature-tagged mutagenesis, we identified a *P. aeruginosa* STM5437 mutant with an insertion into the PA5437 gene (called pycR for putative pyruvate carboxylase regulator). PycR inactivation results in 100 000-fold attenuation of virulence in the rat lung *in vivo*. PycR has the signature of a transcriptional regulator with a predicted helix–turn–helix motif binding to a typical LysR DNA binding site in the PA5436 (pycA)–PA5437 (pycR) intercistronic region. Two pyruvate carboxylase subunits (pycA and pycB) are divergently transcribed upstream of pycR. Transcriptional start sites of pycR and pycA are located at −127 and −88 bp upstream of their initiation codons with Shine–Dalgarno and putative promoter sequences containing −10 and −35 sequences. The DNA binding of PycR was confirmed by DNA mobility shift assay. Genome-wide transcriptional profiling and quantitative real-time PCR (qRT-PCR) indicated that the genes differentially regulated by PycR include two pyruvate carboxylase genes and genes necessary for lipid metabolism, lipolytic activity, anaerobic respiration and biofilm formation. PycR is a regulator with pleiotropic effects on virulence factors, such as lipase and esterase expression and biofilm formation, which are important for maintenance of *P. aeruginosa* in chronic lung infection.

INTRODUCTION

The versatile and ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* causes infection in immunocompromised individuals and in cystic fibrosis (CF) patients, and is highly resistant to antibiotics (Rukholm et al., 2006). The pathogen produces a large variety of both cell-associated and extracellular virulence factors. Pathogenesis of *P. aeruginosa* is not defined by a single virulence factor, but by the precise and delicate interplay between different highly regulated factors, leading to efficient colonization and biofilm formation, tissue necrosis, invasion and dissemination through the vascular system, as well as activation of both local and systemic inflammatory responses. Despite detailed knowledge of extracellular and several surface-associated proteins, many virulence factors essential for pathogenicity, and the mechanisms by which they coordinate function during pathogenesis, remain to be elucidated.

There are at least 498 genes encoding transcriptional regulators or putative regulators in the genome of *P. aeruginosa* (www.pseudomonas.com), and the biological functions of most of these regulators remain largely uncharacterized. There are 125 putative LysR-type transcriptional regulators (LTTRs), which are similarly sized,
autoregulatory proteins that presumably evolved from a distant common ancestor, diverging into subfamilies found in diverse prokaryotic genera (Schell, 1993). Several P. aeruginosa virulence genes have been shown to be under the control of LTRs. For example, MfpR positively regulates the production of elastase and phospholipase, autoinducers, homoserine lactone (PAI I), Pseudomonas quinolone signal (PQS) and phenazine biosynthesis (Wade et al., 2005). Exotoxin A production in P. aeruginosa is a highly regulated process that involves several genes, including the LTR PtXR (Carty et al., 2003). P. aeruginosa strains infecting patients with CF acquire a mucoid phenotype due to overproduction of alginate, and the key enzyme in alginate synthesis is AlgD, which has been shown to be under the control of the LTR CysB (Deliattree et al., 1997). The LTRs from other bacterial pathogens have also been found to be implicated in transcriptional regulation of various virulence and antibiotic resistance factors. For example, AphA is a quorum sensing (QS)-regulated activator that initiates the virulence cascade in Vibrio cholerae by cooperating with the LTR AphB (Kovacicova et al., 2005).

Using an insertional mutation-based screening method for the simultaneous identification of virulence genes important for in vivo maintenance, we have identified in preliminary screening 148 virulence-associated genes encoding 137 novel and 11 previously identified virulence factors (Potvin et al., 2003). As is the case for all high-throughput signature-tagged mutagenesis (STM) screenings, the challenge of mutants identified as defective in virulence lies in defining the function of the corresponding genes. In this study, we have characterized P. aeruginosa PycR encoded by PA5437 as a regulator of virulence factors in vivo. PycR belongs to the LTR family and is essential for maintenance of P. aeruginosa in a rat model of chronic lung infection. Inactivation of pycR abolishes the expression of several virulence factors, including lipase, esterase and biofilm production.

**METHODS**

**Bacterial strains, plasmids, primers, media and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, both Escherichia coli and P. aeruginosa were grown in tryptic soy broth (TSB) or Mueller–Hinton broth (MHB) (Difco). When needed, these media were supplemented with 1.5% bacto agar, gentamicin (Gm; 50 µg ml⁻¹ for *PycR*: Gmª mutant and PA01/pUCP19: Gmª, or 150 µg ml⁻¹ for *P. aeruginosa* exconjugants), ampicillin (Ap; 100 µg ml⁻¹ for *E. coli* SM10), chloramphenicol (Cm; 34 µg ml⁻¹ for *E. coli* Tuner(DE3)pLacI], tetracycline (Tc; 10 µg ml⁻¹ for *E. coli* S17-1 or 50 µg ml⁻¹ for *P. aeruginosa* (Sigma–Aldrich) or carbencillin [Cb; 50 µg ml⁻¹ for *E. coli* Tuner(DE3)pLacI or 300 µg ml⁻¹ for *P. aeruginosa*] (Invitrogen). Primers used in this study are listed in Supplementary Table S1. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase and T4 polynucleotide kinase were purchased from New England Biolabs and used in standard procedures (Sambrook & Russell, 2001). HotStart Taq DNA polymerase was from Qiagen and the PCRs were performed in an iCycler (Bio-Rad).

**Construction of the P. aeruginosa mutant *ΔpycR*.** For construction of deletion mutant P. aeruginosa *ΔpycR*, the sacl-based strategy described elsewhere was used (Hoang et al., 1998). Plasmid pMON3500 (pUCP19 containing the pycR ORF; see below for methods for complementation of the *ΔpycR* mutation) was double-digested with BbsI and BssHII to delete 430 bp of pycR and gel-purified using a Perfectprep Gel Cleanup kit (Eppendorf Canada). Truncated pMON3500 was then blunted using 1.5 U T4 DNA polymerase, 0.1 mM dNTPs, BSA and buffer as recommended by the manufacturer in a total volume of 20 µl, and was purified using Amicon-Microcon-PCR Filter Devices (Millipore). The DNA of the blunt Gmª selectable marker flanked by Flp recombinease target sites was then ligated into the truncated pMON3500 and introduced by electroporation into *E. coli DH10B* (Table 1). To obtain the insert from the vector pUCP19, double digestion with Xmal and SflI was performed, and the insert (208 bp) was gel-purified, blunted and purified using Microcon-PCR. A 2 kb truncated pMON3500 + Gmª was cloned into the Smal site of pEX18Tc and transformed into *E. coli* S17-1 (Table 1). The recombinant plasmids were conjugated from *E. coli* S17-1 into PA01 at 30 °C at a 1:10 recipient: donor ratio, and exconjugants were selected on *Pseudomonas* isolation agar (PIA; Difco) containing 150 µg Gm ml⁻¹. Merodiploids were resolved by plating on PIA medium containing 150 µg Gm ml⁻¹ and 5% sucrose. Deletion of the chromosomally integrated Gmª marker by Flp recombinease-catalysed excision was achieved by conjugal transfer of the PEP-expressing pFLP2 vector from *E. coli* SM10 (Table 1) into *P. aeruginosa* PA01/pycR::Gmª at 30 °C. *P. aeruginosa* PA01/pycR::Gmª and PA01/pycR mutants were confirmed by performing colony PCR on several isolates using PA5437-Xmal and PA5437-SacI primers (Supplementary Table S1). Cell suspensions of PIA-grown sucroseR/Tc sensC/sensG mR or TcS/sensC sensG mS cells were boiled for 5 min, and lysates were then transferred to tubes containing PCR buffer (Qiagen), 200 µM dNTPs and 10 pmol of each primer with 2.5 U HotStart Taq DNA polymerase (Qiagen). Touchdown PCR (65–55 °C) was performed. The PA01/pycR::Gmª mutant strain was then used for in vivo experiments and the *ΔpycR* mutant strain for all other virulence experiments.

**Complementation of *pycR* mutation.** Plasmid pMON3500 (Table 1) was constructed using an *Xmal–SacI* fragment (1.2 kb) from *P. aeruginosa* PA01 containing the ORF of the *pycR* gene amplified by PCR and cloned into the multiple cloning site of the vector pPEL18Tc. Vector DNA was gel-purified. Amplification and restriction sites were introduced by PCR using 10 pmol of two 30-mer oligonucleotides, PA5437-Xmal and PA5437-SacI (Supplementary Table S1), with 2.5 U HotStart Taq DNA polymerase, 200 µM dNTPs and buffer as recommended by the manufacturer, with genomic DNA of *P. aeruginosa* PA01. Touchdown PCR (70–60 °C) was performed as described above. PCR products were purified using Microcon-PCR. Plasmid pMON3500 (pUCP19 containing *pycR* ORF) was introduced by electroporation into *E. coli* DH10B (Table 1), confirmed by sequencing, and electroporated into *P. aeruginosa* *ΔpycR* mutant.

**Primer extension analysis.** RNA was isolated from exponentially growing cells (OD₆₀₀=0.6) (Bowtell & Sambrook, 2003) of *P. aeruginosa* strain PA01 containing the plasmid pMON3500 (pUCP19 containing pycR ORF). RNA was quantified using a QuantiT (Perkin–Elmer Life Sciences.). Primer extension reactions were carried out using 60 µg RNA and the Primer Extension System--AMV Reverse Transcriptase kit (Promega). Primer extension reactions were electrophoresed on a sequencing gel.
along with DNA sequencing reactions performed with the same primers that were used for the primer extension reactions. Sequencing reactions were completed using a Sequenase PCR Product Sequencing kit (USB).

**Cloning and overproduction of PycR protein.** For construction of the C-terminal His-tagged PycR expression vector pM3N501, the pycR gene was amplified from *P. aeruginosa* PA01 genomic DNA by PCR. The primers were 38-mer oligonucleotide PA5437_for and PA5437_rev (Supplementary Table S1). The resulting PCR product was digested with NcoI and 30-mer oligonucleotide PA5437_m for cloning into the multiple cloning site of pETBlue-2 (Novagen). Plasmid pM3N501 (pETBlue-2 containing pycR ORF) was introduced by electroporation into *E. coli* DH10B (Table 1), and the integrity of the pycR gene was confirmed by sequencing. For PycR overexpression, clones of *E. coli* Tuner(DE3)pLacI (Novagen) carrying pM3N501 were grown at 37°C with shaking in LB broth (EMD Chemicals) containing 50 μgCb ml⁻¹ and 34 μgCm ml⁻¹. At OD₆₀₀ 0.5, the culture was induced with 0.01 mM IPTG and incubated overnight at 32°C with shaking, after which cells were harvested by centrifugation and resuspended in 4 ml PycR buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) containing 1 mg lysozyme ml⁻¹ (Sigma–Aldrich) and protease inhibitor cocktail tablets (Complete Mini, EDTA-free, Roche Diagnostics). Cells were broken at 4°C by sonication, and insoluble material was removed by centrifugation at 4°C for 30 min at 17 000 r.p.m. The identity of the PycR protein was confirmed by Western blotting using anti-His-tag antibody and by MALDI-TOF MS.

**Electrophoretic mobility shift assay (EMSA).** The EMSA was based on a protocol of Schuster et al. (2004). A specific DNA probe of ~241 bp was generated by PCR amplification of the intergenic region between pycA and the pycR genes using the PA5436-37_for and PA5436-37_rev primers (Supplementary Table S1). A non-specific DNA probe was generated by PCR amplification of ~177 bp of the pucP19 cloning vector replaced with 956 bp Xbal fragment of GmR selectable marker lacking FLP recombinase target (FRT) sites from pUC18, as a specific competitor. Loading dye (1 : 30 bis-acrylamide to acrylamide ratio) in 0.5 TBE buffer at 6°C was added to 10 μl PCR reaction mixtures, and samples were loaded on 5% native polyacrylamide gels based on a protocol of Schuster et al. (2004). After electrophoresis, the gels were dried on filter paper and exposed to PhosphorImager technology.
Preparation of agarose bead-embedded bacteria for in vivo experiments. The preparation protocol for *P. aeruginosa* wild-type/mutant bacterial mixture in agarose beads was modified from that of van Heekeren & Schluchter (2002). PA01 wild-type containing plasmid pUCP19 or pUCP19::GmR (Table1), the ΔApypR::GmR mutant and the complemented ΔApypR mutant were grown separately in TSB. After overnight incubation in a shaking incubator at 37 °C, the OD₆₀₀ of each culture was noted. A 100 μl aliquot of each overnight culture was diluted to 10 ml with fresh TSB to give a final concentration of ~1 x 10⁷ c.f.u. ml⁻¹. A 250 μl aliquot of the wild-type was mixed with 250 μl of the mutant and added to 4.5 ml TSB. A 200 ml volume of sterile mineral oil was equilibrated at 48 °C and vigorously agitated in a water bath. A 20 ml volume of 2% low-melting-temperature agarose (NuSieve, FMC BioProduct) in PBS was also prewarmed at 48 °C and rapidly mixed with a 5 ml final volume of an equal ratio of wild-type and mutant mixture and added to the mineral oil. The final cell concentration was estimated to be ~1 x 10⁶ c.f.u. ml⁻¹, and at the end of the preparation we expected an approximate bacterial loss of 2 logs. The mixture was cooled gradually for 5 min with ice chips. The agarose beads were washed with 200 ml 0.5% deoxycholic acid sodium salt (SDC, Sigma–Aldrich) in PBS, once with 0.25% SDC/PBS and three times with PBS in a 500 ml Squibb-type separator funnel. The bead slurry was allowed to settle for a few minutes at 4 °C and the remaining PBS was removed. The agarose beads were then homogenized for 30 s with a polytron homogenizer (Kinematica, model PTA 20S, Dispergier und Mischtechnik) and serially diluted. Dilutions were plated in triplicate on Mueller–Hinton agar (MHA) plates with appropriate antibiotics (500 μg Cb ml⁻¹ for wild-type PA01/pUCP19 or complemented ΔapypR mutant selection, and 50 μg Gm ml⁻¹ for ΔApypR::GmR mutant or wild-type PA01/pUCP19::GmR selection).

In vivo and in vitro competitive index (CI). We have used the rat infection model for determination of the in vivo CI of ΔApypR::GmR and the complemented ΔApypR mutant strains. Male Sprague–Dawley rats, 450–500 g in weight, were used in accordance with the rules of the ethics committee for animal treatment. The animals were anaesthetized using isoflurane and inoculated by intubation with 120 μl of a suspension of agar beads containing 10⁹ c.f.u. wild-type/mutant bacterial mixture. After 7 days, lungs were removed from sacrificed rats, and homogenized tissues were plated in c.f.u. wild-type/mutant bacterial mixture. After 7 days, lungs were homogenized and plated on agar plates and colonies were counted. The ratio of wild-type to mutant in each lung was calculated and the competitive index (CI) was calculated as follows: CI = (c.f.u. wild-type/c.f.u. mutant) / (c.f.u. wild-type/c.f.u. mutant). The CI data were represented as the geometric mean for each group.

Biofilm formation assay. To assess and quantify the formation of biofilm in the two wild-type strains PA01 and PA14 and in PA01ApypR, complemented ΔApypR mutant and PA14Δapd-36 mutant, a 96-well-plate rapid biofilm formation assay was performed as described elsewhere (O’Toole & Kolter, 1998a). All strains were grown in the presence of 1 mM IPTG in overnight LB cultures.

Sampling, RNA extraction and transcriptional profiling. For GeneChip analysis, *P. aeruginosa* PA01 wild-type and ΔapypR mutant were grown in TSB broth at 37 °C with shaking. Samples (10 ml) of cells from early exponential phase cultures (OD₆₀₀=0.4) were collected, and DNA degradation was minimized by adding 1.25 ml ice-cold 5% (v/v) phenol in absolute ethanol (pH <7.0). RNA was extracted and purified using a standard procedure (Bowtell & Sambrook, 2003). DNA contamination of purified RNA was monitored using PCR for amplification of the rplU gene with the primers rplU_for and rplU_rev (Supplementary Table S1). RNA integrity was monitored by agarose gel electrophoresis of glyoxylated samples. Preparation of labelled cDNA and processing of the *P. aeruginosa* GeneChip arrays was performed as described elsewhere (Schuster et al., 2003). Washing, staining, and scanning of the GeneChips were performed by the University of Iowa DNA Core Facility using an Affymetrix fluidics station. GeneChip data after two repeats were analysed using GeneChip Operating Software (Affymetrix). Analysis of all results was performed using the *P. aeruginosa* Genome Project database website (www.pseudomonas.com), protein–protein BLAST (blastp) and the conserved domain architecture retrieval tool (CDART) on the NCBI server (http://www.ncbi.nlm.nih.gov/).

Isolation of total RNA, synthesis of cDNA and RT-PCR analysis. In order to validate the results obtained by GeneChip analysis, seven differentially expressed genes were also tested by quantitative real-time PCR (qRT-PCR) with total RNA. *P. aeruginosa* PA01 wild-type and ΔApypR mutant were prepared in the same way as described for transcriptional profiling. Total RNA extraction was performed using the TRIzol method (Invitrogen) according to the instructions of the supplier; then the RNA cleanup was done using an RNeasy Mini kit (Qiagen). cDNA was generated from 1 μg total RNA using a random primer hexamer (100 ng μl⁻¹, Invitrogen) and a QuantitiTec Reverse Transcription kit with integrated removal of genomic DNA contamination (Qiagen) following the instructions provided by the manufacturer. The cDNA mixture was diluted 1:10 in water prior to real-time PCR quantification. A cDNA equivalent to 10 ng total RNA was used per 15 μl PCR reaction. Amplifications were performed in 1× Quantitect SYBR Green mixture (Qiagen) with 0.3 μM 5’ and 3’ primers. Primer design was performed with Primer3 (Rozen & Skaltsky, 2000), available at http://bioitools.umassmed.edu/bioapps/primer3 www.cgi, and primers are listed in Supplementary Table S1 (from PA0171-for to PA5436-rev). All primers had a melting temperature (Tₘ) between 62 and 67 °C, and all amplicon sizes were between 100 and 250 bp. Amplifications were performed in a LightCycler 480 (Roche Diagnostics). After an initial 15 min activation step at 95 °C, 45 cycles (94 °C for 10 s, 62 °C for 2 min) were performed, and a single fluorescent reading was obtained after each cycle immediately following the annealing/elongation step at...
family transcriptional regulator of the CO$_2$-binding operons. The structural organization of pycR and the divergently transcribed PA5436–5435 (pycAB) operon is shown in Fig. 1. pycR has signature motifs of a helix–turn–helix DNA binding region, and LysR motifs were predicted with sequences from www.pseudomonas.com using BLASTP and CDART software from the NCBI server (http://www.ncbi.nlm.nih.gov/).

Frequently, LTTRs are divergently transcribed from their target genes (Schell, 1993). As depicted in Fig. 1, P. aeruginosa pycAB, encoding pyruvate carboxylase subunits (Lai et al., 2006), are divergently transcribed from pycR, suggesting that pycR controls their expression.

**Construction of PAO1 ΔpycR mutant and complementation of the ΔpycR mutation**

In order to eliminate possible polar effects due to insertional inactivation by mini-Tn5 and to obtain a clean genetic background for all in vivo and in vitro experiments, the PAO1ΔpycR deletion mutant was constructed by allelic replacement (Hoang et al., 1998). The ΔpycR deletion was confirmed by PCR (data not shown). For complementation analysis, an Xmal–SacI 1.2 kb genomic DNA fragment containing the complete pycR gene was cloned into the expression vector pUCP19 (see Methods) and transformed into the PAO1ΔpycR mutant strain.

**In vivo importance of P. aeruginosa PycR in chronic lung infection**

Since the STM5437 mutant was identified as being attenuated in vivo in combination with 72 other STM mutants in the pool, we further confirmed the in vivo defect of the ΔpycR mutant in competition with the wild-type strain PAO1. The PAO1ΔpycR mutant and complemented strain were tested in rat lungs for 7 days to estimate in vivo maintenance in comparison with the wild-type strain. As depicted in Fig. 2, a mutation in pycR caused a severe defect.
The mutant in glucose minimal medium.

Partial growth deficiency of the P. aeruginosa in growth and maintenance. The PAO1 mutant in glucose minimal medium.

Fig. 2. In vivo CI of P. aeruginosa ΔpycR::GmR and complemented ΔpycR mutant after 7 days in the rat lung in competition with the wild-type PAO1 strain. Each circle represents the CI for a single animal in each group. A CI of less than 1 indicates a virulence defect. Open circles indicate that no mutant was recovered from the animal, and 1 was substituted in the numerator when calculating the CI. The geometric mean of the CIs for all rats was 2.96 and 0.97, respectively. The PAO1 strain. The growth of all strains tested was similar on TSA or LB media (data not shown).

in growth and maintenance in vivo. The PAO1ΔpycR mutant strain gave a significant 100 000-fold decrease in c.f.u. in rat lung tissues. We noted that four animals had no bacteria 7 days post-infection. In contrast, the complemented mutant strain could be maintained, although growth was not completely restored to the level of the wild-type PAO1 strain. The partial in vivo complementation was expected because pycR gene expression of the complemented strain is under the control of the E. coli lac promoter present on the plasmid vector, which could not be induced in vitro.

Furthermore, we confirmed that ΔpycR and the complemented mutant strain grew at the same rate as the wild-type PAO1 by using an in vitro CI. In comparison with the wild-type strain PAO1, the average in vitro CIs for PAO1ΔpycR and for the complemented mutant strain were 2.96 and 0.97, respectively.

Characterization of the P. aeruginosa ΔpycR mutant

Partial growth deficiency of the P. aeruginosa ΔpycR mutant in glucose minimal medium. We noted a growth lag for the STM5437 mutant in minimal M9 (Gibco, BRL) medium supplemented with 0.4% glucose, 2 mM MgSO4 and 0.1 mM CaCl2. To eliminate the possibility of auxotrophy, we compared the growth in minimal liquid media (M9) of P. aeruginosa STM5437, the STM5437 complemented strain, and the ΔpycR and the complemented ΔpycR strains with the growth of the wild-type strain PAO1 (data not shown). A slight delay in growth was observed for the ΔpycR (and STM5437) mutant at the beginning of the exponential phase. However, the mutant caught up with the wild-type after 6 h, and at 24 h, the numbers of c.f.u. were similar to that of the wild-type strain. P. aeruginosa ΔpycR (and STM5437) complemented in trans grew like the wild-type PAO1 strain. The growth of all strains tested was similar on TSA or LB media (data not shown).

Influence of the ΔpycR mutation on biofilm formation. Since the P. aeruginosa ΔpycR mutant showed reduced virulence in the lung infection model and since biofilm formation is crucial for bacterial maintenance, we tested in vitro biofilm formation. The ΔpycR mutant and the complemented strain were tested for their ability to form a biofilm on an abiotic surface in polystyrene dishes, and compared with the wild-type strain PAO1. Bacteria were grown in the wells in minimal M63 medium supplemented with glucose (0.2%), MgSO4 (1 mM) and casamino acids (CAA; 0.5%) (O’Toole & Kolter, 1998b). The surface attachment-defective PA14sad-36 mutant was used as a negative control. The growth rate in minimal medium after 6 h of incubation was the same for all strains tested (data not shown). The PAO1ΔpycR mutant was defective for biofilm formation since the crystal violet-stained ring formed on walls of PVC wells was smaller when compared with the wild-type strain (data not shown). Quantification of biofilm shown in Fig. 3(c) indicated that the number of biofilm-forming cells was significantly lower for the PAO1ΔpycR mutant in comparison with wild-type PAO1. In contrast, the

Reduced lipolytic activity of P. aeruginosa ΔpycR mutant. Following the confirmation of the reduced in vivo virulence of the ΔpycR mutant, several in vitro phenotypes known to be important for virulence were also examined. We tested H2O2 sensitivity, heat shock, proteolytic activity and bacterial motility of the ΔpycR mutant. These phenotypes were found to be similar to those of the wild-type strain. In contrast, the lipolytic activities of P. aeruginosa ΔpycR were altered. For both lipase (Fig. 3a) and esterase (Fig. 3b), the ΔpycR mutant showed significantly reduced activities. Complementation of the ΔpycR mutant restored the wild-type phenotype in both assays, confirming the efficacy of PycR expression when inducer is used. These results suggested that PycR potentially modulates these lipolytic activities. Phospholipase C production was slightly, but not significantly, lower for the ΔpycR mutant strain when compared with the wild-type PAO1 (data not shown).

P. aeruginosa transcriptional regulator PycR
complemented ΔpycR mutant restored biofilm production to the level of the wild-type PAO1 strain in the presence of inducer.

**Analysis of the divergently transcribed operon pycAB**

**Identification of the transcriptional start site of pycA and pycR genes.** To identify the transcriptional mRNA start site for pycR, a primer extension analysis was done using total RNA extracted from the P. aeruginosa strain PAO1 containing pMON3500 (see Methods). Fig. 4 (a, b) shows that the extended products mapped at nucleotide positions −88 and −127 upstream of the GTG/ATG initiation codons of the pycA and pycR genes, respectively. The pycA −10 (ATCCCA) and −35 (AACAAAT) and pycR −10 (TCGAGG) and −35 (TATAAG) sequences are indicated by boxes in Fig. 4(c). In both cases, the sequences showed three mismatches for −10 and two and one mismatches, respectively, for −35 (mismatches indicated in bold type) in comparison with the E. coli sigma 70 consensus −10 (TTGACA) and −35 (TATAAT) (deHaseth et al., 1998).

LTTRs generally bind to inverted nucleotide repeats containing a T-N_{11}-A motif in the core sequence (Schell, 1993). An examination of the sequence upstream of the pycR transcriptional start site revealed three typical LysR motifs (Fig. 4c), adjacent to its −10/−35 region. The second PycR motif (indicated by bold italic type in Fig. 4c) had a perfect inverted repeat (CTGC-N_{7}-GGAG), and the other two motifs had three and two mismatches, respectively. Three of those motifs were also found in the pycA −10/−35-adjacent regions (Fig. 4c).

**Confirmation of the DNA binding capacity of PycR.** The pycR gene from P. aeruginosa was placed under the control of the T7 promoter of the pETBlue-2 expression vector and transformed into E. coli Tuner(DE3)pLacI. Overproduction of a 34.6 kDa protein at 37 °C resulted in extensive inclusion body sequestration of PycR protein after IPTG induction (data not shown). Efforts to resolubilize biologically active protein were not successful; however, lowering the temperature to 32 °C during expression resulted in low-level production of active soluble PycR. The identity of the PycR protein was confirmed by MALDI-TOF MS and by Western blotting using anti-His-tag antibody (data not shown). The 241 bp PCR fragment between pycA and pycR was amplified and used for EMSA with cell extracts of E. coli Tuner(DE3)pLacI harbouring pMON3501 (pETBlue-2/ pycR) (Fig. 5).

The first retarded band representing DNA−protein binding activity was detected in a gel-shift assay containing different concentrations of PycR extracts (Fig. 5, lanes 1−3). In this case, the shifting of bands was obtained with an excess of PycR protein. However, we noted a reduction of intensity of the binding complex with lower

**Fig. 3.** Phenotypic characterization of the ΔpycR mutant. Lipolytic activities of P. aeruginosa wild-type PAO1, ΔpycR, complemented ΔpycR mutant, lipase-negative mutant ΔlipA/H and esterase-negative ΔestA mutant. Relative lipolytic activities of cell-free culture supernatants in liquid assays were determined as the ratio of OD_{410} to OD_{600} per ml culture. Lipase (a) and esterase (b) activities were assayed with p-nitrophenyl palmitate and p-nitrophenyl caproate, respectively. (c) Quantification of biofilm formation for P. aeruginosa wild-type PAO1, PAO1ΔpycR mutant, complemented PAO1ΔpycR mutant and wild-type PA14 and PA14sad-36 mutant. Statistically significant values obtained with the unpaired t test (P<0.05) are indicated with asterisks for comparisons with the wild-type (PAO1 or PA14), and with # for comparisons with the complemented ΔpycR mutant.
concentrations of PycR (at 5.4 μg protein extract; Fig. 5, lane 3), which suggested concentration-dependent binding. The second migrating band obtained in the gel-shift assay was probably non-specific, because it was weakly detectable in protein extracts from the control strains containing the plasmid vector only (Fig. 5, lanes 9–10). In addition, the increased intensity of this band with lower PycR protein concentrations indicated that this complex was non-specific and due probably to other proteins present in the crude E. coli extract. To confirm PycR binding specificity in the first complex, a chase experiment using an unlabelled specific DNA probe was used in a competition assay and mixed with labelled DNA. As depicted in Fig. 5, lanes 4–6, the decrease in the intensity of the signal correlated with the increase in unlabelled probe acting as a specific competitor. These data indicated that only the first complex is specific for PycR binding when sufficient amounts of specific DNA and PycR are provided for optimal complex formation and equilibrium. No binding activity was detected in a binding assay without protein (Fig. 5, lane 7). The first binding complex was observed with non-induced culture (Fig. 5, lane 8), indicating a basal level of PycR expression. Since almost all LysR-like regulators act in the presence of a coinducer, either PycR interacts with specific DNA in the absence of a coinducer or the coinducer was present in E. coli cell extracts. This could also explain the absence of binding activity when using purified PycR.

Microarray and qRT-PCR analysis of genes regulated by PycR

To evaluate the role of PycR in P. aeruginosa global gene expression, we compared the transcriptomes of mid-exponential phase (OD₆₀₀=0.4) PAO1 and ΔpycR strains using Affymetrix GeneChips. As listed in Table 2, the ΔpycR mutant strain showed 34 genes affected in their expression; 17 genes were found to be upregulated and 17 genes were repressed. We noted that the most important changes were in the transcription levels of pycA and pycB, encoding two pyruvate carboxylase subunits, providing evidence that pycR regulates these two genes. These genes were repressed 25.7 and 27.5-fold, respectively in the ΔpycR mutant strain (hence, these genes would be induced in the wild-type by PycR). These data confirm that PycR regulates these two divergently transcribed genes. To validate the results of GeneChip analysis, we tested, by qRT-PCR, the expression of six genes found to be repressed and one gene found to be upregulated. As shown in Table 2, we obtained the same expression patterns with slightly lower expression fold changes between PAO1 and ΔpycR strains.

Influence of PycR regulation on overall metabolic activities

A metabolomics analysis of the P. aeruginosa ΔpycR mutant strain was done using PMs, which provide a 2D array
Fig. 5. EMSA demonstrating PycR expression in E. coli and its DNA binding activity to the pycA–pycR intergenic region. Crude protein extracts prepared from E. coli Tuner(DE3)pLacI carrying pMON3501 (pETBlue-2/pycR) were examined for PycR DNA binding activity in the presence of 100 pM specific and non-specific DNA probes with 43.2, 32.4 and 5.4 μg PycR protein extract (lanes 1–3). Binding assays in lanes 4–6 were performed in the presence of unlabelled specific competitor DNA. These samples contained 100 pM specific DNA probe with 64.8, 48.6 and 43.2 μg PycR protein extract using 4.7, 23.5 and 28.2 ng unlabelled specific competitor DNA, respectively. Lane 7 contained no protein and 241 bp specific and 177 bp non-specific free DNA probes. Extracts from non-induced culture (lane 8) and protein extracts from E. coli containing the pETBlue-2 plasmid before (lane 9) or after IPTG induction (lane 10) served as controls.

technology for analysis of live cells to measure hundreds of cellular properties simultaneously (Bochner et al., 2001). As shown in Table 3, the most important changes were observed on PM1 and PM2 plates measuring the catabolic functions for carbon utilization. In PM1, the P. aeruginosa ΔpycR mutant strain had lost its capacity to metabolize N-acetyl-d-glucosamine, potentially involved in biofilm formation, as well as glycerol, x-ketobutyric acid, x-hydroxybutyric acid, acetoclastic acid and citric acid. There were no differences between the PAO1 wild-type and the PAO1 ΔpycR mutant strains on PM4 plates measuring phosphorus and sulfur utilization.

**DISCUSSION**

We report here that inactivation of the LysR-type transcriptional regulator encoded by PA5437 (now called PycR) caused serious attenuation of P. aeruginosa in vivo and had an effect on the expression of several virulence factors, such as lipolytic activity and biofilm formation. We also showed that PycR participates in regulation of genes implicated in fatty acid biosynthesis, in bacterial response to anaerobiosis and nitrate utilization, in biofilm formation and in the modulation of genes regulated by QS.

Transcriptomics analysis and qRT-PCR confirmed that PycR is required for expression of the divergently transcribed pycAB genes, which encode z4/4-type pyruvate carboxylase subunits (Lai et al., 2006). Pyruvate carboxylase (PYC) is an ecologically, medically and industrially important enzyme. PYC catalyses ATP-dependent carboxylation of pyruvate to oxaloacetate, and is responsible for replenishing oxaloacetate for continued operation of the tricarboxylic acid cycle (Jitrapakdee & Wallace, 1999; Segura & Espin, 2004). Also, it serves gluconeogenic, glycerogenic and anaerobic roles, which are often vital for cell survival (Branson et al., 2002). Most bacterial and all eukaryotic PYCs are of the z4 type, and each subunit carries both the PYCA and PYCB domains (Jitrapakdee & Wallace, 2001). Specific activities were minimal when cells were grown on casamino acids, acetate or succinate, but were three to fourfold higher when cells were grown in glucose, gluconate, glycerol, lactate or pyruvate minimal media. This enzymic reaction is dependent on pyruvate, ATP and Mg2+, but is not affected by either the presence or the absence of acetyl CoA (Phibbs et al., 1974). The partial growth deficiency of the P. aeruginosa ΔpycR mutant in glucose minimal media suggests the involvement of PycR in PYC regulation.

Lipases are enzymes that catalyse both the formation and the cleavage of long-chain acylglycerols (Jaeger et al., 1999), and they are important in pathogenicity and biofilm formation (Stehr et al., 2003). PMs showed that the P. aeruginosa ΔpycR mutant cannot utilize glycerol, x-ketobutyric, x-hydroxybutyric, acetoclastic and citric acids. In vitro experiments and transcriptomics have demonstrated that the pycR mutation has some effects on lipolytic activities, and may cause fatty acid biosynthesis (FAB) perturbation and the accumulation of long-chain fatty acids, which repress lipA/H expression (Rosenau & Jaeger, 2000). In transcriptomics analysis, PA4200, homologous to the YtnP lipase of Bacillus subtilis, was repressed by PycR. The pycR mutation can be complemented in trans and can fully restore lipolytic activity, indicating its importance in modulation of some virulence determinants.

Only moderate effects of PycR mutation on biofilm formation were demonstrated by in vitro experiments. GeneChip analysis showed that five genes influenced by PycR, PA0049, PA0170, PA0171, PA2130 and PA3540, were implicated in biofilm formation. The PycR activation of PA0171 was also confirmed by qRT-PCR. PA0171 has been shown to be involved in twitching motility (Shan et al., 2004) and to be under AlgR activation during the mid-exponential phase of growth (Lizewski et al., 2004).
The PA2130 gene, activated by PycR and recently designated cupA, encodes a probable fimbrial biogenesis usher protein, part of the gene cluster predicted to encode a novel fimbrial adhesin involved in biofilm formation (Vallet et al., 2001). This system was found to be induced during anaerobic growth with nitrate (see Supplementary Fig. S1) (Filiatrault et al., 2005). In this study, PA0049 and PA3540 were repressed by PycR. The hypothetical PA0049 was induced in developing biofilms (Waite et al., 2005) and repressed during anaerobic growth (Filiatrault et al., 2005). PA3540 GDP-mannose 6-dehydrogenase AlgD was induced by mucoidy and by confluent biofilm (Firoved et al., 2004; Waite et al., 2005). Furthermore, metabolomics has identified that the ΔpycR mutant cannot use N-acetyl-D-glucosamine, although its capacity to use D-galactose is enhanced. Both compounds are important for biofilm formation. D-Galactose is known to reverse the coaggregation of some oral bacteria (Kolenbrander & Andersen, 1989).

The viability of P. aeruginosa robust anaerobic biofilms requires rhl QS and nitric oxide (NO) reductase to modulate or prevent accumulation of toxic NO, a byproduct of anaerobic respiration (Yoon et al., 2002). NO reductase has an important role in the protection of pathogenic bacteria against NO, a toxic free radical produced by macrophages to kill invading micro-organisms (Baek et al., 2004). A group of genes shown to be regulated

<table>
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<tr>
<th>Gene</th>
<th>Description</th>
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<tr>
<td>PA0049</td>
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<td>algD, GDP-mannose 6-dehydrogenase</td>
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<td>PA0523</td>
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Table 2. Global analysis using Affymetrix microarrays and qRT-PCR of genes differentially regulated by the P. aeruginosa PycR transcriptional regulator

Affymetrix microarrays and qRT-PCR assays were done in duplicate using RNA from mid-exponential phase cells. Statistically significant (P≤0.05) values obtained with the unpaired t test for qRT-PCR analysis are indicated with asterisks.
by PycR are implicated in anaerobic respiration, denitrification and nitrogen metabolism, and are QS regulated. Three genes activated by PycR were NO reductase subunits C and B (norCB (PA0523 and PA0524), which perform denitrification and participate in protection against nitrosative stress (Philippot, 2005), and the nitrous-oxide reductase (nosZ (PA3392), which reduces nitrate (NO\_3^-) and nitrite (NO\_2^-) to NO, nitrous oxide (N\_2O) and dinitrogen (N\_2) (Arai et al., 2003). The differential expression of these three genes (PA0523, PA0524 and PA3392) was confirmed by qRT-PCR, but the differences in fold changes between wild-type and D\_pycR mutant were not significant in qRT-PCR experiments. Furthermore, the pycAB gene products have been shown to be repressed by nitrate (Supplementary Fig. S1) (Filiatrault et al., 2005).

The PA2109–PA2114 operon was repressed by PycR. The repression of PA2114 expression was confirmed by qRT-PCR (Table 2). PA2114 encodes a probable major facilitator superfamily (MFS) transporter and has recently been shown to be upregulated in developing biofilms (Waite et al., 2005). In contrast, PA2110 and PA2112–2114 were found to be repressed during anaerobic growth with nitrate, and PA2110, PA2112 and PA2114 were induced in cultures grown aerobically with or without nitrate (Filiatrault et al., 2005). PMs of the P. aeruginosa D\_pycR mutant also demonstrated that three metabolic pathways involved in nitrogen utilization were induced: the utilization of L-asparagine, \(\beta\)-phenylethylamine and D-galactosamine.

This study provides evidence that the P. aeruginosa transcriptional regulator PycR is necessary for in vivo maintenance, has an indirect effect on virulence factors such as lipase/esterase and biofilm production, and modulates the expression of genes implicated in lipid metabolism, anaerobic respiration, biofilm formation and the expression of some QS-regulated genes (Supplementary Fig. S1). PycR positively controls the expression of two subunits of pyruvate carboxylase A and B (PA5435–5436). The NO reductase (norB, C) and nitrous-oxide reductase (nosZ) genes, genes involved in biofilm formation, and some QS-regulated genes were also moderately induced by PycR. PycR represses the genes potentially implicated in anaerobic respiration, lipolytic activity, and nitrogen and lipid metabolism. Thus, PycR is an important regulator, which plays a role in P. aeruginosa lung pathogenesis and represents a potential target for development of novel metabolic-type antimicrobials.

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