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 coordinately 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 LTRRs. For example, MvfR 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 LTRR 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 LTTR CysB (Delic-Attree et al., 1997). The LTRRs 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 LTTR AphB (Kovacikova 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 LTTR 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 *ApyCR::Gm\(^{\ast}\) mutant and PAO1/pUCP19; Gm\(^{\ast}\); 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* SI7-1 or 50 μg ml\(^{-1}\) for *P. aeruginosa* Sigma–Aldrich) or carbenicillin [Cb; 50 μg ml\(^{-1}\) for *E. coli Tuner(DE3)pLacI or 500 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 ApyCR.** For construction of deletion mutant *P. aeruginosa* ApyCR, the sacbI-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 ApyCR 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 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-ended Gm\(^{\ast}\) selectable marker flanked by Flp recombinase 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 *Xma*I and *Sfi*I was performed, and the insert (2038 bp) was gel-purified, blunt-ended and purified using Microcon-PCR. A 2 kb truncated pMON3500 + Gm\(^{\ast}\) was cloned into the Smal site of pEX18Tc and transformed into *E. coli* SI7-1 (Table 1). The recombinant plasmids were conjugated from *E. coli* SI7-1 into PAO1 at 30 °C at a 1:1 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 Gm\(^{\ast}\) marker by Flp recombinase-catalysed excision was achieved by conjugal transfer of the Flp-expressing pFLP2 vector from *E. coli* SM10 (Table 1) into *P. aeruginosa* PAO1ApyCR; Gm\(^{\ast}\) at 30 °C. *P. aeruginosa* PAO1ApyCR; Gm\(^{\ast}\) and PAO1ApyCR mutants were confirmed by performing colony PCR on several isolates using PAS437-Xmal and PAS437-SacI primers (Supplementary Table S1). Cell suspensions of PIA-grown sucrose<sup>6</sup>, Tc<sup>2</sup>, Cb<sup>3</sup>, Gm<sup>4</sup> or Tc<sup>3</sup>, Cb<sup>3</sup>, Gm<sup>4</sup> 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, and the PAO1ApyCR; Gm<sup>4</sup> mutant strain was then used for *in vivo* experiments and the ApyCR mutant strain for all other virulence experiments.

**Complementation of ApyCR mutation.** Plasmid pMON3500 (Table 1) was constructed using an *Xmal–SacI* fragment (1.2 kb) from *P. aeruginosa* PAO1 containing the ORF of the pycR gene amplified by PCR and cloned into the multiple cloning site of the shuttle vector pUCP19. Vector DNA was gel-purified. Amplification and restriction sites were introduced by PCR using 10 pmol of two 30-mer oligonucleotides, PAS437-Xmal and PAS437-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* PAO1. 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* ApyCR mutant.

**Primer extension analysis.** RNA was isolated from exponentially growing cells (OD<sub>600</sub> = 0.6) (Bowtell & Sambrook, 2003) of *P. aeruginosa* strain PAO1 containing plasmid pMON3500 (pUCP19 containing pycR ORF). RNA was quantified using a Quant-IT RiboGreen RNA assay kit (Molecular Probes, Invitrogen). The primers used in extension experiments were PAS436-37_for and PAS436-37_rev (Supplementary Table S1), which correspond to nucleotides −4 to +10 and −4 to +14 relative to the PAS436 (pycA) and pycR initiation codons, respectively. Primers were radioactively labelled using [γ-<sup>32</sup>P]ATP (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.
Table 1. Bacterial strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
<td>ElectroMaxDH10B</td>
<td>Electrocompetent cells</td>
<td>Invitrogen</td>
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<td>S17-1 (pir)</td>
<td>Tp&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, RP4-2-Tc::Mu-Km::Tn7, recA deletion of <em>E. coli</em> 294</td>
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<tr>
<td>SM10</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu</td>
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<td>Tuner(DE3)pLacI</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, lacZY deletion mutant of BL21</td>
<td>Novagen</td>
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<td><strong>P. aeruginosa strains</strong></td>
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<td>PAO1</td>
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<td>PAO1 STM5437::miniTn5 mutant inactivating PA5437 gene</td>
<td>Potvin et al. (2003)</td>
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<td>Wilhelm et al. (1999)</td>
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<tr>
<td>PASCH1</td>
<td>PAO1ΔestA, mutant defective in esterase</td>
<td>Wilhelm et al. (1999)</td>
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<td>PAO1293ΔpycR::Gm&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;, 430 bp replacement of PA5437 (pycR) gene with Gm&lt;sup&gt;R&lt;/sup&gt; cassette</td>
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<td>PA14</td>
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<td>PA14sad-36</td>
<td>PA14sad-36 (figK)::Tn5B30, Tc&lt;sup&gt;R&lt;/sup&gt;, biofilm mutant</td>
<td>O’Toole &amp; Kolter (1998b)</td>
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<td><strong>Plasmids</strong></td>
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<td>Cb&lt;sup&gt;R&lt;/sup&gt;, cloning vector</td>
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<td>pUCP19::Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Gm&lt;sup&gt;R&lt;/sup&gt;, 743 bp Solp/Bsal fragment of bla gene of pUCP19 cloning vector replaced with 956 bp Xbal fragment of Gm&lt;sup&gt;R&lt;/sup&gt; selectable marker lacking FLP recombinase target (FRT) sites</td>
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<td>Gb&lt;sup&gt;R&lt;/sup&gt;, PA5437 (pycR) gene in pUCP19 vector</td>
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<td>pEX18Tc</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;, ori&lt;sup&gt;T&lt;/sup&gt; sac&lt;sup&gt;B&lt;/sup&gt;&lt;sup&gt;R&lt;/sup&gt;, gene replacement vector with multiple cloning site (MCS) from pUC18</td>
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<td>pMON3501</td>
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<td>This study</td>
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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 pMON3501, the *pycR* gene was amplified from *P. aeruginosa* PAO1 genomic DNA by PCR. The primers were 38-mer oligonucleotide PA5437_Ncol and 30-mer oligonucleotide PA5437_XhoI (Supplementary Table S1) and 70–60 °C Touchdown PCR was performed as described above. The resulting PCR product was digested with Ncol and XhoI, purified using Microcon-PCR and cloned into the multiple cloning site of expression vector pETBlue-2 (Novagen). Plasmid pMON3501 (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 pMON3501 were grown at 37 °C with shaking in LB broth (EMD Chemicals) containing 30 µg Cb ml<sup>-1</sup> and 34 µg Cm ml<sup>-1</sup>. At OD<sub>600</sub> 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, 10 mM NaCl, 0.1 mM DTT) containing 1 mg lysosome ml<sup>-1</sup> (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 *pycR* gene in pUCP19 vector (Supplementary Table S1). The resulting PCR products were labelled using [γ-<sup>32</sup>P]ATP (Perkin–Elmer) and T4 polynucleotide kinase. Binding reactions contained 100 pmol of each specific and non-specific DNA in a final volume of 20 µl DNA-binding buffer [20 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 100 µg BSA ml<sup>-1</sup>, 10 µg poly(dIdC) ml<sup>-1</sup>, 5 % (v/v) glycerol]. Cell extracts of *E. coli* Tuner(DE3)pLacI harbouring pETBlue-2 alone or pMON3501 (pETBlue-2 containing the *pycR* ORF), before and after IPTG induction, were added at the indicated concentrations and the reaction mixtures were incubated at room temperature for 30 min. The unlabelled specific DNA was used at the indicated concentrations as a specific competitor. Loading dye [250 mM Tris-HCl, pH 7.5, 0.2 % (w/v) bromophenol blue, 40 % (v/v) glycerol] was added to control reactions only, and the reaction mixtures were electrophoresed on native 5 % Tris-borate-EDTA (TBE) polyacrylamide gels (1:30 bis-acrylamide to acrylamide ratio) in 0.5 × TBE buffer at 150 V for 1–2 h at room temperature. The gel was subsequently dried, and radio-labelled protein–DNA complexes were detected using Phosphorimager technology.
Preparation of agaroze bead-embedded bacteria for in vivo experiments. The preparation protocol for *P. aeruginosa* wild-type/mutant bacterial mixture in agaroze beads was modified from that of van Heekeren & Schluchter (2002). PA01 wild-type containing plasmid pUCP19 or pUCP19::Gm<sup>R</sup> (Table 1), the ΔpycR::Gm<sup>R</sup> mutant and the complemented ΔpycR mutant were grown separately in TSB. After overnight incubation in a shaking incubator at 37 °C, the OD<sub>600</sub> 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 × 10<sup>6</sup> c.f.u. ml<sup>-1</sup>. 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 × 10<sup>6</sup> c.f.u. ml<sup>-1</sup>, and at the end of the preparation we expected an approximate bacterial loss of 2 logs. The mixture was cooled gradually for 48 h, and 1 ml of cell culture was centrifuged for 5 min at 9000 g. The agaroze 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 Squivb-type separator funnel. The bead slurry was allowed to settle for a few minutes at 4 °C and the remaining PBS was removed. The agaroze 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 (MAH) plates with appropriate antibiotics (500 μg Cb ml<sup>-1</sup> for wild-type PA01/pUCP19 or complemented ΔpycR mutant selection, and 50 μg Gm ml<sup>-1</sup> for ΔpycR::Gm<sup>R</sup> mutant or wild-type PA01/pUCP19::Gm<sup>R</sup> selection).

**In vivo and in vitro competitive index (CI).** We have used the rat infection model to determine the in vivo CI of ΔpycR::Gm<sup>R</sup> and the complemented ΔpycR 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 isofluorane and inoculated by intubation with 120 μl of a suspension of agar beads containing 10<sup>7</sup> c.f.u. wild-type/mutant bacterial mixture. After 7 days, lungs were removed from sacrificed rats, and homogenized tissues were plated in triplicate on PIA for assay of the total number of *P. aeruginosa* cells from early exponential phase cultures (OD<sub>600</sub> = 0.4) were collected, and RNA 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 rpsL gene with the primers rpsL_for and rpsL_rev (Supplementary Table S1). RNA integrity was monitored by agaroze 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 ΔpycR 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<sup>-1</sup>, Invitrogen) and a QuantiTec 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 & Skaletsky, 2000), available at http://biotools.umassmed.edu/biopatts/primer3 www.cgi, and primers are listed in Supplementary Table S1 (from PA0171-for to PA3436-rev). All primers had a melting temperature (T<sub>mel</sub>) between 62 and 67 °C, and all amplicon sizes were between 100 and 250 bp. Amplifications were carried out 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 72 °C.

**Biofilm formation assay.** To assess and quantify the formation of biofilm in the two wild-type strains PA01 and PA14 and in PAO1ΔpycR, complemented ΔpycR mutant and PA14Δsad-36 mutant, a 96-well-plate rapid biofilm formation assay was performed as described elsewhere (O’Ttoole & 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 ΔpycR mutant were grown in TSB broth at 37 °C with shaking. Samples (10 ml) of cells from early exponential phase cultures (OD<sub>600</sub> = 0.4) were collected, and RNA 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 rpsL gene with the primers rpsL_for and rpsL_rev (Supplementary Table S1). RNA integrity was monitored by agaroze 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/).
62 °C. A melting curve was performed at the end of cycling to ensure that there was single amplification. Crossing point (Cp) values were determined with the software supplied with the instrument. Standard curves were used to transform Cp values obtained from total RNA samples into transcript number. Samples were normalized to the reference gene rpsL (PA4268).

Phenotype MicroArray analysis. To assess the overall effect of PycR transcriptional regulation on cell metabolism, phenotype analyses were carried out in triplicate using Phenotype MicroArrays (PMs) (Biolog) which measure carbon (C) (PM1–2), nitrogen (N) (PM3) and phosphorus (P)/sulfur (S) (PM4) metabolism. This technique involves the deduction of cellular phenotypes using cell respiration as the reporter system. Before inoculation into the PM1, PM2, PM3 and PM4 96-well microplates, PAO1 wild-type and ΔpycR mutant were grown overnight at 37 °C on plates containing the nutrient-limited medium R2A agar. Cells were picked up from the surface of the R2A plates using a sterile cotton swab and were resuspended in IF-0 (inoculating fluid) supplemented with menadione sodium bisulfite (5.524 µg ml⁻¹) for PM1–2 and with menadione sodium bisulfite (5.524 µg ml⁻¹), sodium succinate (5.402 µg ml⁻¹) and ferric citrate (0.49 µg ml⁻¹) for PM3–4 at a density corresponding to 85 % transmittance in the Biolog turbidimeter using 20 mm diameter tubes. The suspensions were then inoculated into the appropriate microplate at a volume of 100 µl per well. The microplates were placed at 37 °C for either 24 h (PM1–2) or 48 h (PM3–4), at which time sufficient purple colour had developed in the positive control wells, while the negative control wells remained colourless. The microplates were then examined for utilization of the C, N, P or S source in a particular well.

Statistical analysis. Statistical analyses were performed with GraphPad Prism 5 software using the unpaired t test or the Mann–Whitney sum test.

RESULTS

Identification of the \textit{P. aeruginosa} STM5437 mutant

Novel pathogenicity genes important for infection of \textit{P. aeruginosa} had been identified previously using PCR-based STM (PCR-STM) (Lehoux et al., 2002, 2004). A collection of 7968 STM mutants was screened in a rat model of chronic lung infection (Cash et al., 1979). A total of 214 mutants, representing transposition events into 148 ORFs, were shown to be attenuated in lung infection and were retained for further analysis (Potvin et al., 2003). The STM5437 mutant inactivating the PA5437 gene was shown to be attenuated in a pool of 72 STM mutants when screened in a rat lung model of chronic infection. These studies indicated a possible attenuation for the STM5437 mutant, which was chosen for detailed analysis.

Analysis of the PA5437 (pycR) gene

The PA5437 gene, now called \textit{pycR}, for putative pyruvate carboxylase regulator, was identified by bioinformatics analysis as a putative transcriptional regulator, which codes for a 311 aa protein of ~34.6 kDa. According to www.pseudomonas.com, the \textit{P. aeruginosa} PycR had 44 % identity with CbbRI of \textit{Rhodobacter capsulatus}, a LysR family transcriptional regulator of the \textit{CO₂}-binding operons. The structural organization of \textit{pycR} and the divergently transcribed PA5436–5435 (\textit{pycAB}) operon is shown in Fig. 1. \textit{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, \textit{P. aeruginosa} \textit{pycAB}, encoding pyruvate carboxylase subunits (Lai et al., 2006), are divergently transcribed from \textit{pycR}, suggesting that \textit{pycR} controls their expression.

Construction of \textit{PAO1ΔpycR} mutant and complementation of the \textit{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 \textit{in vivo} and \textit{in vitro} experiments, the \textit{PAO1ΔpycR} deletion mutant was constructed by allelic replacement (Hoang et al., 1998). The \textit{ΔpycR} deletion was confirmed by PCR (data not shown). For complementation analysis, an \textit{XmaI}–\textit{Sacl} 1.2 kb genomic DNA fragment containing the complete \textit{pycR} gene was cloned into the expression vector pUCP19 (see Methods) and transformed into the \textit{PAO1ΔpycR} mutant strain.

\textit{In vivo} importance of \textit{P. aeruginosa} PycR in chronic lung infection

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

\textbf{Fig. 1.} Genomic organization of the PA5436–5435 (\textit{pycAB}) operon and the PA5437 (\textit{pycR}) gene. The \textit{pycA} and \textit{pycB} genes encode two subunits of pyruvate carboxylase. The \textit{pycR} gene encodes a transcriptional regulator of 311 aa, and motifs such as the helix–turn–helix DNA binding region and LysR motifs were identified by bioinformatics analysis. The arrows indicate the direction of transcription.
in growth and maintenance in vivo. The PAO1 ΔpycR 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 MgSO\(_4\) and 0.1 mM CaCl\(_2\). 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).

**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 H\(_2\)O\(_2\) 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).

**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 polyvinylchloride (PVC) microtitre dishes, and compared with the wild-type strain PAO1. Bacteria were grown in the wells in minimal M63 medium supplemented with glucose (0.2%), MgSO\(_4\) (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
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 pycA (PA5436) and pycR (PA5437) genes, 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 (AACAAT) 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-N11-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 (C\(\uparrow\)T\(\downarrow\)GC-N7-GC\(\uparrow\)A\(\downarrow\)G), 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
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 in 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
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, α-ketobutyric acid, α-hydroxybutyric acid, acetooacetic 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 α4β4-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, glycogenic and anaerobiotic roles, which are often vital for cell survival (Branson et al., 2002). Most bacterial and all eukaryotic PYCs are of the α4 type, and each subunit carries both the PYCA and PYCB domains (Jitrapakdee & Wallace, 1999; Lim et al., 1988; Mukhopadhyay et al., 1998; Samols et al., 1988). Since *P. aeruginosa* PYC is of the α4β4 type and different from eukaryotic α4 PYCs, it represents a potent antimicrobial target. The PYC activity has been detected in cell extracts of *P. aeruginosa* strain PAO (Phibbs et al., 1974). 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, α-ketobutyric, α-hydroxybutyric, acetooacetic 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 these 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 coaggregations 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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<th>Gene</th>
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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\textsubscript{3}) and nitrite (NO\textsubscript{2}) to NO, nitrous oxide (N\textsubscript{2}O) and dinitrogen (N\textsubscript{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 \textit{DpycR} mutant were not significant in qRT-PCR experiments. Furthermore, the \textit{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 \textit{P. aeruginosa DpycR} 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 \textit{P. aeruginosa} transcriptional regulator PycR is necessary for \textit{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 (\textit{norB}, C) and nitrous-oxide reductase (\textit{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 \textit{P. aeruginosa} lung pathogenesis and represents a potential target for development of novel metabolic-type antimicrobials.

### ACKNOWLEDGEMENTS

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### REFERENCES


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Table 3. PM (Biolog) analysis of the \textit{P. aeruginosa DpycR} mutant indicating its capacity to metabolize various carbon and nitrogen sources

<table>
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<td>Saccharic acid</td>
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<tr>
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<td>(\beta)-Phenylethylamine</td>
<td>D-Galactosamine</td>
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</tbody>
</table>

Table 3.

PM (Biolog) analysis of the \textit{P. aeruginosa DpycR} mutant indicating its capacity to metabolize various carbon and nitrogen sources


involved in twitching motility in *Pseudomonas aeruginosa*. *Microbiology* 150, 2653–2661.


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