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.

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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,

Abbreviations: CF, cystic fibrosis; CI, competitive index; LTTR, LysR-type transcriptional regulator; MFS transporter, major facilitator superfamily transporter; PM, Phenotype MicroArray; qRT-PCR, quantitative real-time PCR; QS, quorum sensing; STM, signature-tagged mutagenesis.

A supplementary table listing the PCR primers used and a supplementary figure showing a transcriptome analysis of genes differentially regulated by *P. aeruginosa* PycR are available with the online version of this paper.

The microarray data for this paper have been deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) with accession number E-MEXP-1591.

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 LTTRs. 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 LTTR 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 LTTRs 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 Δ pycR::Gm^R mutant and PAO1/pUCP19::Gm^R, 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 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 Δ pycR. For construction of deletion mutant *P. aeruginosa* Δ pycR, the *sacB*-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 Δ pycR mutation) was double-digested with *Bbs*I and *Bss*HII 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 blunted Gm^R 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, blunted and purified using Microcon-PCR. A 2 kb truncated pMON3500 + Gm^R was cloned into the *Sma*I site of pEX18Tc and transformed into *E. coli* S17-1 (Table 1). The recombinant plasmids were conjugated from *E. coli* S17-1 into PAO1 at 30 °C at a 10:1 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^R 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* PAO1 Δ pycR::Gm^R at 30 °C. *P. aeruginosa* PAO1 Δ pycR::Gm^R and PAO1 Δ pycR mutants were confirmed by performing colony PCR on several isolates using PA5437-*Xma*I and PA5437-*Sac*I primers (Supplementary Table S1). Cell suspensions of PIA-grown sucrose^R, Tc^S, Cb^S, Gm^R or Tc^S, Cb^S, Gm^S 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 PAO1 Δ pycR::Gm^R mutant strain was then used for *in vivo* experiments and the Δ pycR mutant strain for all other virulence experiments.

Complementation of Δ pycR mutation. Plasmid pMON3500 (Table 1) was constructed using an *Xma*I–*Sac*I 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, PA5437-*Xma*I and PA5437-*Sac*I (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* Δ pycR mutant.

Primer extension analysis. RNA was isolated from exponentially growing cells (OD₆₀₀=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 PA5436-37_for and PA5436-37_rev (Supplementary Table S1), which correspond to nucleotides –4 to +10 and –4 to +14 relative to the PA5436 (*pycA*) and *pycR* initiation codons, respectively. Primers were radioactively labelled using [γ -³²P]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

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>E. coli</i> strains		
ElectroMaxDH10B	Electrocompetent cells	Invitrogen
S17-1 (λ pir)	Tp ^R , Sm ^R , RP4-2-Tc::Mu-Km::Tn7, <i>recA</i> deletion of <i>E. coli</i> 294	Simon <i>et al.</i> (1983)
SM10	Ap ^R , <i>thi-1 thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu	Hoang <i>et al.</i> (1998)
Tuner(DE3)pLacI	Cm ^R , <i>lacZY</i> deletion mutant of BL21	Novagen
<i>P. aeruginosa</i> strains		
PAO1	PAO1293, Cm ^S , <i>E79 tv-2</i> , wild-type, derivative of prototrophic PAO1	Holloway <i>et al.</i> (1979)
STM5437	PAO1 STM5437::miniTn5 mutant inactivating PA5437 gene	Potvin <i>et al.</i> (2003)
PABS1	PAO1 Δ <i>lipA</i> – Δ <i>lipH</i> , mutant defective in lipase	Wilhelm <i>et al.</i> (1999)
PASCH1	PAO1 Δ <i>estA</i> , mutant defective in esterase	Wilhelm <i>et al.</i> (1999)
PAO1 Δ <i>pycR</i>	PAO1293 Δ <i>pycR</i> mutant, 960 bp fragment with internal deletion of PA5437 (<i>pycR</i>) gene	This study
PAO1 Δ <i>pycR</i> ::Gm ^R	PAO1293 Δ <i>pycR</i> ::Gm ^R , Gm ^R , 430 bp replacement of PA5437 (<i>pycR</i>) gene with Gm ^R cassette	This study
PA14	Wild-type prototrophic laboratory strain	Holloway <i>et al.</i> (1979)
PA14sad-36	PA14sad-36 (<i>flgK</i>)::Tn5B30, Tc ^R , biofilm mutant	O'Toole & Kolter (1998b)
Plasmids		
pUCP19	Cb ^R , cloning vector	West <i>et al.</i> (1994)
pUCP19::Gm ^R	Gm ^R , 743 bp <i>SspI/BsaI</i> fragment of <i>bla</i> gene of pUCP19 cloning vector replaced with 956 bp <i>XbaI</i> fragment of Gm ^R selectable marker lacking FLP recombinase target (FRT) sites	This study
pMON3500	Cb ^R , PA5437 (<i>pycR</i>) gene in pUCP19 vector	This study
pEX18Tc	Tc ^R , <i>oriT</i> ⁺ <i>sacB</i> ⁺ , gene replacement vector with multiple cloning site (MCS) from pUC18	Hoang <i>et al.</i> (1998)
pFLP2	Ap ^R , 2.6 kb <i>BamHI/SphI</i> fragment from pALB2 ligated between the same sites of pPS908	Hoang <i>et al.</i> (1998)
pETBlue-2	Cb ^R , R6K-based suicide delivery plasmid	Novagen
pMON3501	Cb ^R , PA5437 (<i>pycR</i>) gene in pETBlue-2 vector	This study

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_*NcoI* 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 *NcoI* 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 50 μ g Cb ml⁻¹ and 34 μ g Cm 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, 10 mM NaCl, 0.1 mM DTT) containing 1 mg lysosyme 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 *pycR*-coding region using the PA5437_cod_for and PA5437_cod_rev primers (Supplementary Table S1). The resulting PCR products were labelled using [γ -³²P]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 μ l DNA-binding buffer [20 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 100 μ g BSA ml⁻¹, 10 μ g poly(dIdC) ml⁻¹, 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 $\times$ TBE buffer at 150 V for 1–2 h at room temperature. The gel was subsequently dried, and radiolabelled protein–DNA complexes were detected using 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 Heeckeren & Schluchter (2002). PAO1 wild-type containing plasmid pUCP19 or pUCP19::Gm^R (Table 1), the $\Delta pycR::Gm^R$ mutant and the complemented $\Delta pycR$ 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 $\sim 1 \times 10^9$ 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 $\sim 1 \times 10^6$ 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 PAO1/pUCP19 or complemented $\Delta pycR$ mutant selection, and 50 µg Gm ml⁻¹ for $\Delta pycR::Gm^R$ mutant or wild-type PAO1/pUCP19::Gm^R selection).

***In vivo* and *in vitro* competitive index (CI).** We have used the rat chronic lung infection model to determine the *in vivo* CI of $\Delta pycR::Gm^R$ and the complemented $\Delta pycR$ 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 10^4 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* bacterial cells and on MHA supplemented with 500 µg Cb ml⁻¹ or 50 µg Gm ml⁻¹. In conjunction with each *in vivo* competition, an *in vitro* competition of $\Delta pycR::Gm^R$ and complemented mutant was carried out with the same bacterial ratios. First, the bacterial mixtures were stored overnight at 4 °C in 25 ml TSB and then grown to late-exponential phase at 37 °C with shaking. Competitive indexes (CIs) were calculated as the ratio of mutant to wild-type bacteria recovered *in vivo* (or from *in vitro* bacterial mixtures) adjusted by the input ratio. The final CI data were represented as the geometric mean for animals in the same group. Each *in vivo* and *in vitro* competition was tested for statistical significance by Student's two-tailed *t* test.

Lipase and esterase assays. Bacteria were grown aerobically in glass tubes in 2 ml LB broth in the presence of 1 mM IPTG at 37 °C for 48 h, and 1 ml of cell culture was centrifuged for 5 min at maximum speed. Lipase and esterase extracellular activities for wild-type PAO1, the $\Delta pycR$ mutant and, as respective controls, the PABS1 lipase-defective mutant and the PASCH1 esterase-defective mutant (Table 1) were determined from cell-free culture supernatants. For the lipase quantitative assay, 30 mg *p*-nitrophenyl palmitate (pNPP; Sigma-Aldrich) was dissolved in 10 ml 2-propanol and added to 90 ml Sørensen phosphate buffer (17 parts 63 mM Na₂HPO₄ to one part 5 mM KH₂PO₄; pH 8.0) (K. E. Jaeger, personal communication) supplemented with 207 mg sodium deoxycholate and 100 mg gum arabic, yielding a final pNPP concentration of 1 mM. For the esterase

assay, 2.15 µl 1 mg *p*-nitrophenyl caproate ml⁻¹ (pNPC=*n*-hexanoic acid 4-nitrophenyl ester; ABCR) was dissolved in ethanol (5 ml), and added to tubes with 9.5 ml 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgSO₄ to yield a final concentration of 0.1 mM pNPC. For both assays, samples (80 µl) were added to the substrate solution to give a final volume of 1 ml, and the $\Delta OD_{410} \text{ min}^{-1}$ was recorded for 5–15 min at 37 °C for lipase and at room temperature for esterase with a Cary spectrophotometer (Varian). The relative enzyme activities were determined as the ratio OD₄₁₀ (enzyme activity) to OD₆₀₀ (cell density).

Biofilm formation assay. To assess and quantify the formation of biofilm in the two wild-type strains PAO1 and PA14 and in PAO1 $\Delta pycR$, complemented $\Delta pycR$ mutant and PA14sad-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* PAO1 wild-type and $\Delta pycR$ 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 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 *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* PAO1 wild-type and $\Delta pycR$ 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 QuantiTect 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/bioapps/primer3_www.cgi, and primers are listed in Supplementary Table S1 (from PA0171-for to PA5436-rev). All primers had a melting temperature (*T_m*) 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

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 $\Delta pycR$ 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 $\mu\text{g ml}^{-1}$) for PM1–2 and with menadione sodium bisulfite (5.524 $\mu\text{g ml}^{-1}$), sodium succinate (5.402 $\mu\text{g ml}^{-1}$) and ferric citrate (0.49 $\mu\text{g ml}^{-1}$) 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 *P. aeruginosa* STM5437 mutant

Novel pathogenicity genes important for infection of *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 *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 *P. aeruginosa* PycR had 44 % identity with CbbRI of *Rhodobacter capsulatus*, a LysR

family transcriptional regulator of the CO₂-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 $\Delta pycR$ mutant and complementation of the $\Delta pycR$ 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 PAO1 $\Delta pycR$ deletion mutant was constructed by allelic replacement (Hoang *et al.*, 1998). The $\Delta pycR$ deletion was confirmed by PCR (data not shown). For complementation analysis, an *Xma*I–*Sac*I 1.2 kb genomic DNA fragment containing the complete *pycR* gene was cloned into the expression vector pUCP19 (see Methods) and transformed into the PAO1 $\Delta pycR$ 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 $\Delta pycR$ mutant in competition with the wild-type strain PAO1. The PAO1 $\Delta pycR$ 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

Fig. 1. Genomic organization of the PA5436–5435 (*pycAB*) operon and the PA5437 (*pycR*) gene. The *pycA* and *pycB* genes encode two subunits of pyruvate carboxylase. The *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.

Fig. 2. *In vivo* CI of *P. aeruginosa* $\Delta pycR::Gm^R$ and complemented $\Delta pycR$ 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 is shown as a solid line and statistically significant values are indicated with an asterisk (* $P=0.0001$, Mann–Whitney sum test). The *in vitro* CIs were 2.96 for $\Delta pycR::Gm^R$ and 0.97 for the complemented $\Delta pycR$ mutant.

in growth and maintenance *in vivo*. The PAO1 $\Delta pycR$ 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 vivo*.

Furthermore, we confirmed that $\Delta pycR$ 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 PAO1 $\Delta pycR$ and for the complemented mutant strain were 2.96 and 0.97, respectively.

Characterization of the *P. aeruginosa* $\Delta pycR$ mutant

Partial growth deficiency of the *P. aeruginosa* $\Delta pycR$ 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 $\Delta pycR$ and the complemented $\Delta pycR$ strains with the growth of the wild-type strain PAO1 (data not shown). A slight delay in growth was observed for the $\Delta pycR$ (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* $\Delta pycR$ (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* $\Delta pycR$ mutant. Following the confirmation of the reduced *in vivo* virulence of the $\Delta pycR$ mutant, several *in vitro* phenotypes known to be important for virulence were also examined. We tested H_2O_2 sensitivity, heat shock, proteolytic activity and bacterial motility of the $\Delta pycR$ mutant. These phenotypes were found to be similar to those of the wild-type strain. In contrast, the lipolytic activities of *P. aeruginosa* $\Delta pycR$ were altered. For both lipase (Fig. 3a) and esterase (Fig. 3b), the $\Delta pycR$ mutant showed significantly reduced activities. Complementation of the $\Delta pycR$ 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 $\Delta pycR$ mutant strain when compared with the wild-type PAO1 (data not shown).

Influence of the $\Delta pycR$ mutation on biofilm formation. Since the *P. aeruginosa* $\Delta pycR$ 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 $\Delta pycR$ 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 PAO1 $\Delta pycR$ 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 PAO1 $\Delta pycR$ mutant in comparison with wild-type PAO1. In contrast, the

Fig. 3. Phenotypic characterization of the $\Delta pycR$ mutant. Lipolytic activities of *P. aeruginosa* wild-type PAO1, $\Delta pycR$, complemented $\Delta pycR$ mutant, lipase-negative mutant $\Delta lipA/H$ and esterase-negative $\Delta estA$ mutant. Relative lipolytic activities of cell-free culture supernatants in liquid assays were determined as the ratio of OD₄₁₀ to OD₆₀₀ 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, PAO1 $\Delta pycR$ mutant, complemented PAO1 $\Delta pycR$ mutant and wild-type PA14 and PA14sad-36 mutant. Statistically significant values obtained with the unpaired *t* test ($P \leq 0.05$) are indicated with asterisks for comparisons with the wild-type (PAO1 or PA14), and with # for comparisons with the complemented $\Delta pycR$ mutant.

complemented $\Delta pycR$ 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* (PA5437) and for the divergently transcribed *pycAB* (PA5436–PA5435) operon, 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 (CTGC-N₇-GCAG), 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. 4. Determination of the *pycA* (a) and *pycR* (b) transcriptional start sites using primer extension analysis. Lane 1 contains a primer extension completed using RNA from *P. aeruginosa* strain PAO1 containing the pMON3500 plasmid (pUCP19/*pycR*), and sequencing reaction lanes are labelled according to nucleotide (A, C, G or T). The extension products are indicated by the arrow for each gene, corresponding to nucleotides -88 and -127 relative to the *pycA* and *pycR* initiation codons, respectively (c). The directions of *pycA* and *pycR* transcription with their respective start sites (+1) are indicated by arrows, and putative -10 and -35 sequences are boxed. The GTG/ATG of the *pycA* and *pycR* genes, the direction of translation of their respective proteins, and putative ribosome-binding sites (Shine–Dalgarno, SD) are also indicated. A typical LysR motif [(T)-N₁₁-(A)] was found three times in both *pycA* and *pycR* -10/-35 regulatory regions and is indicated by brackets (c). Only the second *pycR* motif had perfect inverted repeats C(T)GC-N₇-GC(A)G (bold italic type), while the two other motifs had two mismatches. A total of 15 putative LysR motifs were found in the whole intergenic sequence (T and A of each motif are in lower-case bold type).

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 Δ *pycR* strains using Affymetrix GeneChips. As listed in Table 2, the Δ *pycR* 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 -5.7 and -7.5-fold, respectively in the Δ *pycR* 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 Δ *pycR* strains.

Influence of PycR regulation on overall metabolic activities

A metabolomics analysis of the *P. aeruginosa* Δ *pycR* 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* Δ *pycR* 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, acetoacetic acid and citric acid. There were no differences between the PAO1 wild-type and the PAO1 Δ *pycR* 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 α 4 β 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 anaplerotic roles, which are often vital for cell survival (Branson *et al.*, 2002). Most bacterial and all eukaryotic PYCs are of the α 4 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 α 4 β 4 type and different from eukaryotic α 4 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 Mg^{2+} , 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* Δ *pycR* 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* Δ *pycR* mutant cannot utilize glycerol, α -ketobutyric, α -hydroxybutyric, acetoacetic 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).

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 ($P \leq 0.05$) values obtained with the unpaired *t* test for qRT-PCR analysis are indicated with asterisks.

Gene	Description	Fold change	
		Affymetrix	qRT-PCR
PA0049	Hypothetical protein	+2.9	
PA0262	Conserved hypothetical protein	+3.8	
PA0361	Probable gamma-glutamyltranspeptidase precursor	+2.3	
PA0704	Probable amidase	+3.0	
PA1391	Probable glycosyl transferase	+2.1	
PA2109	Hypothetical protein	+8.7	
PA2110	Hypothetical protein	+5.3	
PA2111	Hypothetical protein	+5.2	
PA2112	Conserved hypothetical protein	+6.2	
PA2113	Probable porin	+4.3	
PA2114	Probable MFS transporter	+6.1	+4.7*
PA2116	Conserved hypothetical protein	+3.8	
PA2372	Hypothetical protein	+2.5	
PA2449	Probable transcriptional regulator	+2.9	
PA3540	<i>algD</i> , GDP-mannose 6-dehydrogenase	+2.0	
PA4200	Hypothetical protein	+3.9	
PA4724	Probable aminoacyl-tRNA synthetase (class I)	+2.7	
PA0170	Hypothetical protein	-3.3	
PA0171	Hypothetical protein	-3.2	-1.8*
PA0523	<i>norC</i> , NO reductase subunit C	-3.4	-2.0
PA0524	<i>norB</i> , NO reductase subunit B	-3.8	-2.6
PA0894	Hypothetical protein	-2.4	
PA1740	Hypothetical protein	-2.5	
PA2130	Probable fimbrial biogenesis usher protein	-2.5	
PA2171	Hypothetical protein	-2.3	
PA2189	Hypothetical protein	-2.7	
PA2209	Hypothetical protein	-2.3	
PA3336	Probable MFS transporter	-2.5	
PA3392	<i>nosZ</i> , nitrous-oxide reductase precursor	-3.1	-1.6
PA4033	Hypothetical protein	-3.4	
PA4155	Hypothetical protein	-2.4	
PA4820	Hypothetical protein	-2.8	
PA5435	<i>pycB</i> , pyruvate carboxylase subunit B	-7.5	-2.9*
PA5436	<i>pycA</i> , pyruvate carboxylase subunit A	-5.7	-2.9*

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 Δ *pycR* 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 3. PM (Biolog) analysis of the *P. aeruginosa* $\Delta pycR$ mutant indicating its capacity to metabolize various carbon and nitrogen sources

Non-metabolized (attenuated) carbon or nitrogen source	Highly metabolized (overused) carbon or nitrogen source
PM1 (carbon)	
N-Acetyl-D-glucosamine	Saccharic acid
Glycerol	D-Galactose
α -Ketobutyric acid	Mucic acid
α -Hydroxybutyric acid	Tricarballic acid
Acetoacetic acid	D-Galacturonic acid
Citric acid	
PM2 (carbon)	
Gelatin	DL-Carnitine
4-Hydroxybenzoic acid	
L-Isoleucine	
PM3 (nitrogen)	
	L-Asparagine
	β -Phenylethylamine
	D-Galactosamine

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 $\Delta pycR$ 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* $\Delta pycR$ mutant also demonstrated that three metabolic pathways involved in nitrogen utilization were induced: the utilization of L-asparagine, β -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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