The PA4204 gene encodes a periplasmic gluconolactonase (PpgL) which is important for fitness of *Pseudomonas aeruginosa*

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In *Pseudomonas aeruginosa*, the PA4204 gene encodes a protein with a signal peptide and a COG2706 domain of the type present in 3-carboxy-*cis*,*cis*-muconate lactonizing enzymes. A molecular model based on the structure of the *Escherichia coli* YbhE phosphogluconate lactonizing enzyme shows that the enzyme has a beta-propeller ('doughnut') structure and a central active site comprising one histidine, one glutamic acid and two arginines. Inactivation of the *P. aeruginosa* PA4204 gene had profound phenotypic effects, resulting in slowly growing small colonies which frequently gave rise to larger colonies. The small colonies did not produce pyocyanin, produced reduced amounts of *N*-acylhomoserine lactones, and had extremely low levels of 2-alkyl-4-quinolones (AQs), while the larger colonies produced pyocyanin and higher amounts of AQs, including the pseudomonas quinolone signal (PQS), compared with the wild-type strain. Mutagenesis of His 182 in PA4204 resulted in the inability of this protein to restore pyocyanin production in the PA4204 isogenic mutant, suggesting that this enzyme may share an active site with other lactonizing enzymes. The protein with signal peptide was expressed as a His fusion in *E. coli* and purified. Two forms were observed, suggesting that the protein is translocated. The purified enzyme cleaved (S)-5-oxo-2-tetrahydrofuran-2-carboxylic acid and D-glucono- δ -lactone, demonstrating lactonase activity. Decreased expression of the cytoplasmic phosphogluconolactonase gene (*pgl*) was observed in the small-colony mutant, and the mutant could not grow in the presence of mannitol or gluconate, suggesting functions in the detoxification of a gluconolactone and in sugar metabolism.

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INTRODUCTION

Fluorescent pseudomonads are ubiquitous Gram-negative γ -Proteobacteria, and can adapt to diverse ecological niches (Goldberg, 2000). Several genomes of fluorescent pseudomonads have been or are being sequenced, reflecting their broad interest to the scientific community

(<http://www.pseudomonas.com>). However, a large proportion of the genes encode proteins of unknown function. This is the case for a gene upstream of *mexGHI-opmD*, PA4204, which encodes a putative periplasmic protein, with a COG2706 domain found in carboxymuconolactonases (CMLEs). The product of PA4204 also has similarity to 6-phosphogluconolactonases (PGLs). PGLs have an important function in the oxidative branch of the pentose phosphate pathway, since they convert δ -6-phosphogluconolactone, the product of the glucose 6-phosphate dehydrogenation, to 6-phosphogluconate, which can enter the Entner–Doudoroff (ED) pathway (Kupor & Fraenkel, 1969; Miclet *et al.*, 2001; Thomason *et al.*, 2004; Zimenkov *et al.*, 2005). In the absence of PGL, δ -6-phosphogluconolactone can spontaneously isomerize to γ -6-phosphogluconolactone, which is toxic (Miclet *et al.*, 2001; Zimenkov

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Abbreviations: AHL, acylhomoserinelactone; AQ, 2-alkyl-4-quinolone; CMLE, carboxymuconolactonase; ED, Entner–Doudoroff; LC-MS, liquid chromatography-mass spectrometry; PGL, phosphogluconolactonase; PQS, pseudomonas quinolone signal; QS, quorum sensing.

A supplementary figure showing the alignment of the product of PA4204 with YbhE of *E. coli*, the L- α -hydroxyglutaric acid gamma-lactonase of *Pectobacterium (Erwinia) cypripedii*, and the CMLE from *Neurospora crassa* is available with the online version of this paper.

et al., 2005). Two kinds of 6-phosphogluconolactonases, which are not structurally similar, have been described: the *Escherichia coli* PGL type, and the *Pseudomonas* type (Hager *et al.*, 2000). Interestingly, the PGL of *Pseudomonas aeruginosa* is a cytoplasmic enzyme encoded by another gene, PA3182, while PA4204 is similar to YhbE, the *E. coli* PGL (Thomason *et al.*, 2004; Zimenkov *et al.*, 2005). PGLs therefore have not only a catalytic function, but also prevent the accumulation of toxic, non-degradable metabolites, having an important 'house-cleaning' function (Galperin *et al.*, 2006). In their article, Galperin *et al.* (2006) indeed describe PA4204 as a possible 6-phosphogluconolactonase with such a house-cleaning function. However, PGLs are normally cytoplasmic, while the product of PA4204 is probably localized in the periplasm. Mochizuki (2003, 2006) identified a similar enzyme, also with a predicted periplasmic location, in *Erwinia cypripedii* 314B (now *Pectobacterium cypripedii*) with a lactonase activity specific for (S)-5-oxo-2-tetrahydrofurancarboxylic acid, which converts this molecule to L- α -hydroxyglutaric acid. Orthologues of the PA4204 gene are also present in many proteobacteria and in all pseudomonads, but not in the same genomic context as in *P. aeruginosa*. In this bacterium, the presence of this gene close to the *mexGH-opmD* locus also raises the question of a possible link with quorum sensing (QS). We have previously shown that mutants in the *mexI* and *opmD* genes produce drastically reduced amounts of the QS signal molecule PQS (pseudomonas quinolone signal) and the phenazine pyocyanin (Diggle *et al.*, 2006; Dietrich *et al.*, 2006; Aendekerk *et al.*, 2002, 2005). We decided to inactivate PA4204 in order to determine whether this gene also influences the production of QS signal molecules. We demonstrate here (i) that the PA4204 gene encodes a periplasmic enzyme with a β -propeller fold; (ii) that it has the same lactonase activity as the enzyme described by Mochizuki (2003, 2006) and that it also cleaves D-glucono- δ -lactone; (iii) that it is important for the fitness of the bacterium and for growth on gluconate, 2-ketogluconate and mannitol; (iv) that in its absence the expression of the cytoplasmic PGL is decreased; and (v) that it affects the production of QS signal molecules, especially the N-acylhomoserinelactones (AHLs) and the alkyl-quinolones, including PQS.

METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids that were used in this study are listed in Table 1. *E. coli* DH5 α and *E. coli* S17-1 λ pir were used for cloning and conjugation experiments. *P. aeruginosa* strains were grown at 37 °C in Luria-Bertani (LB) broth or on LB agar plates, iron-poor casamino acids (CAA) medium (Difco Laboratories), *Pseudomonas* agar medium (Difco Laboratories) or King's B medium (King *et al.*, 1954). The following concentrations of antibiotics were used for *P. aeruginosa*: gentamicin, 100 μ g ml⁻¹; tetracycline, 200 μ g ml⁻¹; chloramphenicol, 350 μ g ml⁻¹. Growth was also followed in minimal succinate medium (Meyer & Abdallah, 1978) containing a limiting amount of succinate (2 mM) and 20 mM of one of the following

carbon sources: phenol, succinate, protocatechuate, *cis,cis*-muconate, catechol, glucose, fructose, gluconate, 2-ketogluconate or mannitol. Because of the appearance of spontaneous pseudo-revertants (see Results), growth was monitored in triplicate and the curves were considered valid for the PA4204 mutant only when growth started at the same moment in the three cultures. Earlier onset of growth in one of the cultures was considered to be the consequence of the appearance of a revertant, and the corresponding data were therefore not taken into account.

DNA manipulation and *in silico* analysis of the PA4204 gene.

Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep kit (Qiagen). All restriction enzymes were purchased from Fermentas and used according to the manufacturer's instructions. DNA subcloning, transformations and agarose gel electrophoresis were done as described elsewhere (Sambrook *et al.*, 1989). Similarity searches were performed with the BLAST program (Altschul *et al.*, 1997) and amino acid sequence alignment was performed using CLUSTAL W (Thompson *et al.*, 1997) or Kalign (Lassmann & Sonnhammer, 2006).

Strain and plasmid construction. An in-frame and unmarked PA4204 deletion mutant of *P. aeruginosa* PAO1 was constructed using the suicide vector pDM4 (Milton *et al.*, 1996). This vector contains the lethal *sacB* gene encoding levansucrase, and recombination of the vector onto the chromosome confers chloramphenicol resistance to the host strain. Approximately 500 bp of upstream and downstream sequences flanking the gene of interest were amplified by PCR with primers PA4204-F and PA4204-R using chromosomal DNA as the template and cloned into pBluescript, resulting in plasmid pBlu4204 (Table 1). These plasmids were used as templates to introduce a deletion by inverse PCR using primers PA4204-UM-F and PA4204-UM-R. The resulting PCR product was digested with *Xma*I and self-ligated, resulting in the plasmid pBlu Δ 4204. The PCR product was excised from the vectors and cloned into the pDM4 vector, resulting in pDM Δ 4204. The pDM4-based deletion allele was mobilized into *P. aeruginosa* and integrated into the chromosome by single crossover using *E. coli* S17-1 λ pir as the delivery strain. Double-crossover events were subsequently selected by growth in the presence of 10% (*P. aeruginosa*) sucrose. Deletion mutants were confirmed by both PCR and sequencing.

Cloning of the PA4204 gene. The complete PA4204 gene was amplified by PCR with primers PA-4204-F and PA-4204-R. The 1.5 kb PCR fragment obtained was digested further with *Clal* and *Bam*HI and cloned into the *Clal*/*Bam*HI-opened vector pBBR1MCS, resulting in pBBR-4204. This plasmid was used to transform *E. coli* DH5 α , and chloramphenicol-resistant colonies were selected. The recombinant plasmid was introduced by electroporation into the PA4204 *P. aeruginosa* mutant.

Molecular modelling. The PA4204 structure was modelled using the automated FFAS03 fold recognition server (Rychlewski *et al.*, 2000) to generate the starting model using *E. coli* YhbE as the template (the YhbE phosphogluconolactonase and CMLE structures gave the highest scores), then manually edited with the molecular graphics program O (Jones *et al.*, 1991) to fix some peptide bonds, and minimized with CNS (Brünger *et al.*, 1998) with conjugate gradient minimization for 100 steps. The structure was then aligned with the *Neurospora crassa* CMLE structure using O to analyse for fit of the conserved residues.

Site-directed mutagenesis using a PCR method with mutagenic primers. The 1.5 kb *Clal*-*Bam*HI fragment containing the entire PA4204 locus was amplified using genomic DNA of *P. aeruginosa* PAO1 and was used for mutagenesis. Mutations were generated via PCR using *Taq* polymerase (Qiagen) with mutagenic

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference or origin
<i>P. aeruginosa</i> strains		
PAO1	Wild-type	ATCC 15692
PAO-S1	PA4204 chromosomal deletion mutant derived from PAO1	This study
PAO-B1	Revertants derived from PAO-S1	This study
PAO-P1	Chromosomal deletion of the <i>pqsA</i> gene in PAO1	Fletcher <i>et al.</i> (2007)
PAO1 $\Delta pqsA$ <i>pqsA::lux</i>	Chromosomal deletion of the <i>pqsA</i> gene in PAO1 containing CTX- <i>lux::pqsA</i>	Fletcher <i>et al.</i> (2007)
<i>E. coli</i> strains		
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i> $\Delta(lacZYA \pm argF)$ U169 (ϕ 80d <i>lacZ</i> Δ M15)	Hanahan (1983)
S17-1 λ pir	<i>thi pro hsdR hsdM+ recA</i> RP4-2-Tc::Mu-Km::Tn7 <i>pir</i>	Simon <i>et al.</i> (1983)
BL21(DE3)	<i>F⁻ ompT gal dcm lon hsdS_B(rB⁻ mB⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</i>	
MH155	<i>E. coli</i> strain harbouring the reporter plasmid pUCP22NotI- P _{lasB} :: <i>gfp</i> (ASV)P _{lac} :: <i>lasR</i>	Hentzer <i>et al.</i> (2002)
<i>Chromobacterium violaceum</i> CV026	Mini-Tn5 mutant of ATCC 31532 deficient in AHL and violacein production	McClellan <i>et al.</i> (1997)
Plasmids		
pBluescript II SK, KS	Cloning vectors, ColE1 replicons; Ap ^r	Stratagene
pET24a(+)	Expression vector	Novagen
pET24SP4	pET24 with PA4204 gene	This study
pDM4	Suicide vector carrying the <i>sacBR</i> genes for sucrose sensitivity (Cm ^r)	Milton <i>et al.</i> (1996)
pBBR1MCS	Wide-host-range cloning vector	Kovach <i>et al.</i> (1994)
pBlu4204	2.125 kb <i>Bgl</i> II PAO1 chromosomal DNA fragment containing PA4204 in pBluescript II SK, KS	This study
pBlu Δ 4204	Same as pBLU::4204, except contains a 768 bp PA4204 deletion in pBluescript II SK, KS	This study
pDM4 Δ 4204	pDM4 containing PA4204 flanking regions and deletion-containing PA4204 gene	This study
pBBR-4204	1.5 kb <i>clal</i> – <i>Bam</i> HI PAO1 chromosomal DNA fragment containing PA4204 in pBBR1MCS	This study
pBBR-4204- Δ H	Same as pBBR-4204; contains a deletion in codon for His 182	This study
pBBR-4204-Ala	Same as pBBR-4204; contains an Ala substitution in codon 182 for His	This study

primers PA-PpgL- Δ H-F, PA-PpgL- Δ H-R, PA-PpgL-Ala-F and PA-PpgL-Ala-R (in which His 182 was deleted or substituted by Ala by changing the CAC codon to GCG) in combination with primers PA-PpgL-F and PA-PpgL-R and the following parameters: 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C for 25 cycles. A final extension of 10 min at 72 °C allowed completion of the PCR fragments. To generate the complete 1.5 kb fragment containing the mutation, a third PCR was performed using *Proofstart* Polymerase (Qiagen) and the products of the former PCRs as template. Primers PA-PpgL-F and PA-PpgL-R were added after five cycles. The nucleotide change was confirmed by sequencing (Forman *et al.*, 2006). Mutated PCR products were digested with *Clal* and *Bam*HI and cloned into pBBR1MCS to generate pBBR-4204- Δ H and pBBR-4204-Ala. These two plasmids were used to complement the PA4204 mutant.

Pyocyanin production. Pyocyanin was visualized by growing the bacteria in LB medium or on P-agar. Pyocyanin was extracted and quantified from stationary-phase cultures as described elsewhere (Mavrodi *et al.*, 2001). All experiments were done in triplicate.

Liquid chromatography-MS (LC-MS) detection of QS signal molecules. For accurate quantification of AHL and 2-alkyl-4-quinolones (AQs), bacterial cultures were grown for 8 h at 37 °C and 200 r.p.m. in 30 ml LB. From each culture, 5 $\times$ 5 ml aliquots were

removed for quintuplicate solvent extractions. Culture supernatants were filtered through a 0.22 μ m pore-size filter and QS molecules were extracted with ethyl acetate, whereas pellets were extracted with methanol and then filtered through a 0.22 μ m pore-size filter. Extracts were dried to completion, resuspended in 1 ml methanol and analysed by reverse-phase HPLC (RP-HPLC) using a Phenomenex Gemini column (C18, 5 μ m, 250 $\times$ 2.00 mm) coupled with MS. The fragmentation ions of each of the anticipated QS molecules were recorded using Multiple Reaction Monitoring (MRM), as used by Ortori *et al.* (2007). Error bars on the relevant figures represent 2 $\times$ SEM (95 % confidence interval).

Cloning, overexpression and purification of PA4204. The complete ORF of PA4204, including part of the signal peptide, was amplified using primers PA4204-SPF (5' GGAATTCCATATG-AGAAGACTGCCACCCTC 3') and PA4204-R1 (5' CCGCTCGAG-CGGCACC GCCACGAAG 3'). Primer PA4204-SPF contains an *Nde*I restriction site, while primer PA4204-R1 contains a *Xho*I restriction site (both underlined). After amplification, the fragment was cloned in the pET24a(+) vector (Novagen) and the ligation mix was used to transform *E. coli* DH5 α , and transformants were selected for their resistance to kanamycin (60 μ g ml⁻¹). Sequencing of the fragment was performed to check for the absence of mutations that could have been generated during the PCR; for this, primers pET24a(+) (5'

CAATTTGTTTTTAATAAAGATC 3') and pET3a-reverse2 (5' CCA-CCGAGTGCACATGAC 3'), and pET3a2 (5' GTCATGTGCACTCG-GTGG 3') and pET3a-reverse (5' CCAACTCAGCTTCCTTCGGGC 3') were used. The recombinant plasmid pET24SP4 was then extracted from *E. coli* DH5 α and used to transform *E. coli* BL21(DE3). Expression of PA4204 in this strain was induced by adding IPTG (1 mM) when the culture reached OD₆₀₀ 0.6 (LB medium). Growth under inducing conditions was done in a 300 ml volume at 30 °C for different periods of time (5 h to overnight). After centrifugation, the cell pellet was resuspended in 10 ml 0.9% (w/v) cold NaCl and centrifuged at 7000 r.p.m. for 10 min. The resulting pellet was then resuspended in 6 ml 20 mM phosphate buffer, 0.5 M NaCl, 40 mM imidazole, pH 7.4. The cells were then broken by sonication in a Vibra Cell sonicator (Fisher Bioblock Scientific) at 20% of the maximal amplitude for 15 min, followed by centrifugation at 10000 r.p.m. for 10 min (Sorvall SS34 rotor), and the supernatant was recovered as a total crude protein extract. The total extract (10 ml) was injected into a His-Trap nickel affinity chromatography column (2 × 1 cm), and the elution was followed in an AKTA FPLC system. First, the column was washed with buffer A (20 mM phosphate buffer, 0.5 M NaCl, 40 mM imidazole, pH 7.4) and the His-tagged protein eluted using buffer B (20 mM phosphate buffer, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The fractions were analysed for their purity by SDS-PAGE (12% gel). The pooled fractions containing the purified proteins were further purified by Superdex 75 gel filtration. The final purified protein peak was then dialysed further overnight against 20 mM PBS, 150 mM NaCl, pH 7.2.

Hydroxyglutaric acid γ -lactonase (HGL) assay. The L- α -hydroxyglutaric acid γ -lactonase (L- α -HGL) activity was determined as described elsewhere (Mochizuki, 2003), with modifications. The activity toward (S)-5-oxo-2-tetrahydrofuran-2-carboxylic acid was determined in an assay mixture containing 10 mM substrate, 10 mM phosphate buffer (pH 7.0), and purified enzyme extract. After incubation at 37 °C for 1–10 min, an aliquot was taken and placed in boiling water for 1 min to terminate the reaction. After removal of the precipitated protein by centrifugation for 10 min at 14000 r.p.m. in an Eppendorf benchtop centrifuge 5417C, the resulting hydroxycarboxylic acid was detected by HPLC as described by Mochizuki (2001) using an Inertsil ODS-3 column (4.6 × 250 mm; GL Science) at a flow rate of 1.0 ml min⁻¹, monitoring at 210 nm with 0.1% (w/v) H₃PO₄ as an eluent. The negative control was assay mixture without enzyme. Protein concentration was determined by standard Bradford method HPLC (Mochizuki, 2001).

Glucono- δ -lactonase assay. Gluconolactonase activity was determined using the colorimetric assay described by Hucho & Wallenfels (1972) and D-glucono- δ -lactone as substrate (Sigma). The substrate solution was prepared immediately before the assay. A 10 ml volume of a 0.25 mM p-nitrophenol (Sigma) solution in 10 mM phosphate buffer (pH 7.2) was added to 18.5 mg D-glucono- δ -lactone (10 mM final concentration). After addition of the enzyme (10 μ l, 1.6 μ g) to 0.99 ml of the mix in the cuvette, the A₄₀₅ was measured at 24 °C. Opening of the lactone ring causes acidification of the medium and a decrease in the absorbance of p-nitrophenol.

RT-PCR analysis of *pgl* gene expression. Bacterial RNA was extracted by using the High Pure RNA Isolation kit (Roche). First-strand cDNA was synthesized by using the First-strand cDNA Synthesis kit (Amersham Biosciences). PCR was performed with the following cycling parameters: 95 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by 72 °C for 10 min. The *oprL* gene was used as a normalizer in this test (Aendekerk *et al.*, 2005). Amplification products were electrophoresed on 0.8% agarose gels (with an 8 μ l loading volume).

RESULTS

In silico analysis of the PA4204 gene

In *P. aeruginosa*, PA4204 is located between PA4203, encoding a putative LysR regulator (upstream, same orientation), and the *mexGHI-opmD* pump genes (downstream, same orientation) (Fig. 1a). The product of PA4204 has 388 amino acids, contains a type I signal peptide with no predicted transmembrane domains and is predicted to have a periplasmic location (Lewenza *et al.*, 2005). A search of the *Pseudomonas* genome databases revealed the presence of PA4204 homologues in *Pseudomonas syringae* B728a (P syr_1712, 51.62% identity at amino acid level), *Pseudomonas fluorescens* Pf5 (PFL_3276, 53.66% identity) and *Pseudomonas putida* KT2440 (PP2021, 50.5% identity), although the genetic context in these organisms was very different to that found in *P. aeruginosa* PAO1 (data not shown).

The predicted PA4204 protein shows similarity with the *E. coli* 6-phosphogluconolactonase YbhE [28% identity and 46% similarity ($E=3.6 \times 10^{-19}$) (gi 16128735)], to the periplasmic lactonase from *Pectobacterium* (*Erwinia*) *cypripedii* (21.5% identity, 36% similarity; gi 83016742; Mochizuki, 2003, 2006) and to the CMLE (18% identity, 35% similarity; P38677) from *Neurospora crassa*, the crystal structure of which has been determined (Mazur *et al.*, 1994; Kajander *et al.*, 2002). Furthermore, the PA4204 protein has the COG2706 domain found in CMLE, but the high similarity to *E. coli* YbhE suggests that it could also be a 6-phosphogluconolactonase. The alignment of the amino acid sequences of these proteins, shown in Supplementary Fig. S1, reveals some conserved residues, including those found to be in the active site of YbhE and the *N. crassa* CMLE (His 182, corresponding to His 148 of *N. crassa* CMLE; Arg 234, corresponding to Arg 196; Glu 250, corresponding to Glu 212; and Arg 302, corresponding to Arg 274). Fig. 1(b) shows the model of the PA4204 encoded protein (without the signal peptide) based on the structure of YbhE from *E. coli* aligned with the *N. crassa* CMLE structure. His 148, Arg 196, Glu 212 and Arg 274 corresponding to the active site of *N. crassa* CMLE are shown in cyan, while the corresponding residues in the *P. aeruginosa* enzyme are shown in green. The rest of the CMLE molecule is omitted for clarity, but aligned perfectly (protein backbones are aligned). The model shows clearly that the enzyme from *P. aeruginosa* has the same β -propeller fold as YbhE and the *N. crassa* CMLE (Kajander *et al.*, 2002).

Phenotypic effects resulting from the inactivation of PA4204 in *P. aeruginosa*

To investigate the role of PA4204 in *P. aeruginosa* an unmarked deletion was generated in this gene using the pDM4 vector (see Methods). The *P. aeruginosa* PA4204 mutant showed a significantly extended lag phase when grown in LB (Fig. 2a, growth monitored in triplicate, see

Fig. 1. (a) Genomic context of the PA4204 gene in *P. aeruginosa* PAO1. (b) Model of the PA4204 protein based on the crystal structure of *E. coli* YbhE phosphogluconolactonase and *N. crassa* CMLE. Residues in the active site of PA4204 are shown in green; those from *N. crassa* CMLE are shown in cyan. The numbering of the active site residues corresponds to the *N. crassa* CMLE (see text for details).

Methods), in relation to the parent, and formed very small colonies after 3 days of incubation at 37 °C (Fig. 2b). On plates, larger colonies appeared at a frequency of 10^{-4} , suggesting the emergence of spontaneous compensatory mutation(s) (Fig. 2b). These spontaneous mutants or large-colony variants showed similar growth to that of the parent (Fig. 2a). Remarkably, the PA4204 deletion resulted in the almost complete loss of the blue-green pigment pyocyanin from the small colonies, whereas the large-colony variants could produce pyocyanin, albeit at lower levels compared with the wild-type (Fig. 3). Complementation of the small-colony mutants with the PA4204 gene resulted in the restoration of wild-type pyocyanin levels (Fig. 3).

Effect of the PA4204 mutation on QS signal molecule production

The observation that the PA4204 small-colony mutant was unable to produce pyocyanin prompted us to look at the levels of QS signal molecules, since the production of this phenazine pigment is controlled by QS via the Rhl system and via the production of PQS and its precursor 2-heptyl-4-quinolone (HHQ) (Gallagher *et al.*, 2002; Dietrich *et al.*, 2006; Price-Whelan *et al.*, 2006; Fletcher *et al.*, 2007; Diggle *et al.*, 2007). As shown in Fig. 4(a), both types of PA4204 mutants showed decreased production of the *N*-acylhomoserine lactones C4-HSL and 3-oxo-C12-HSL, as deter-

mined by quantitative LC-MS analysis. AQ levels, however, were reduced dramatically in the cell-bound fraction as well as in the supernatant of the small-colony PA4204 mutant. Conversely, the levels of these molecules in the large-colony variant were the same or even higher than those in the wild-type PAO1 (Fig. 4b, upper panel corresponding to the cellular fraction, lower panel to the supernatant extracts).

Structure–function analysis of the protein encoded by PA4204

The existence of conserved residues in the active site of the carboxymuconate lactonizing enzyme CMLE and in the gene product of PA4204 (Fig. 1b) suggested that these two proteins may share a conserved active site. To investigate this, we mutagenized the His 184 residue from the *P. aeruginosa* protein, corresponding to His 148 in the CMLE active site, by either deleting it or changing it to an alanine. Fig. 3 shows that a mutation of this residue resulted in the inability of the gene to restore pyocyanin production in the PA4204 isogenic mutant. This shows clearly that the histidine residue predicted to be important for the activity of CMLEs is also important for the activity of the *P. aeruginosa* PA4204 protein. This effect was observed on LB, but was even more pronounced on *Pseudomonas* P agar (data not shown).

Fig. 2. (a) Growth of wild-type *P. aeruginosa* (●), small-colony PA4204 mutant (○), and large-colony revertant (△) in LB medium. Growth was followed in three independent wells and the results considered valid only when the three curves were identical. (b) Appearance of a large-colony revertant of a PA4204 mutant surrounded by the original small-colony type. The large-colony pseudo-revertant colony size is indistinguishable from that of the wild-type.

Cloning, expression and purification of the PA4204 protein

The complete ORF of PA4204 was cloned in the pET24 expression vector, which allows high levels of expression after IPTG induction as well as purification via a C-terminal His-tag. Fig. 5 shows the induction and

purification of the PA4204 protein. After His-trap chromatography and elution of bound proteins with imidazole, two proteins were eluted (Fig. 5a), one with an apparent molecular mass of about 40 kDa, the other of 36–38 kDa. No other band or degradation product was observed. The larger protein was clearly less abundant when the proteins were extracted after 5 h of induction

Fig. 3. Pyocyanin production on LB medium of wild-type (PAO1), PA4204 small-colony mutant (S1), PA4204 large-colony revertant (B1), PA4204 small-colony mutant complemented with PA4204 cloned in pBBR1MCS and lacking His 182, complemented with PA4204 with His 182 replaced by Ala, PA4204 mutant containing the empty vector pBBR1MCS, and PA4204 mutant complemented with the wild-type PA4204 gene. The results are the mean of three experiments; error bars show SD.

Fig. 4. (a) Detection by LC-MS of *N*-acylhomoserine lactones (C4-HSL and 3-oxo-C12-HSL) in supernatant extracts from wild-type PAO1 (black bars), PA4204 small-colony mutant (white bars), and PA4204 large-colony variant (grey bars). The values are the mean of three experiments from five separate extractions; error bars show SD. (b) Upper panel: detection by LC-MS of AQs in cellular extracts of wild-type (black bars), PA4204 small-colony mutant (white bars), and PA4204 large-colony variants (grey bars). HHQ, 2-heptyl-4-quinolone; NHQ, 2-nonyl-4-quinolone; UHQ, 2-undecyl-4-quinolone; HHQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; NHQNO, 2-nonyl-4-hydroxyquinoline-*N*-oxide; PQS, 2-heptyl-3-hydroxy-4-quinolone; C9-PQS, 2-nonyl-3-hydroxy-4-quinolone; C11-PQS, 2-undecyl-3-hydroxy-4-quinolone. Lower panel: similar, but for extracellular extracts.

compared with overnight induction. Western blot analysis showed that both proteins were detected by an anti-His-tag mAb (results not shown). This result is consistent with the processing of the signal peptide of the pre-protein, suggesting a periplasmic localization, as predicted by the presence of a cleavable signal peptide.

Lactonase activity of the PA4204 protein

Since the PA4204 protein showed a high level of similarity to the lactonase of *Pectobacterium (Erwinia) cypripedii* (Mochizuki, 2003, 2006), we examined the activity of the purified protein against the substrate (S)-5-oxo-2-tetrahydrofurancarboxylic acid (Fig. 5b). As shown in Fig. 5(b), the commercial substrate (S)-5-oxo-2-tetrahydrofurancarboxylic acid gives two peaks on HPLC (left panel) and these peaks disappear after incubation with the purified protein fraction (right panel). This result corresponds to what has been described by Mochizuki (2006). Analysis of the different peaks and of the purified substrate [(S)-5-oxo-2-tetrahydrofurancarboxylic acid] and product (L- α -hydroxyglutaric acid) shows that the peak eluting early corresponds to L- α -hydroxyglutaric acid (*m/z* 147). Finally, Fig. 5(c) shows that the purified enzyme has a clear

lactonase activity when D-glucono- δ -lactone is used as substrate. When the enzyme was boiled for 10 min the opening of the gluconolactone occurred at the same rate as without enzyme (spontaneous decay). The enzyme was also partially sensitive to EDTA (Fig. 5c).

Utilization of different carbon sources

Growth was followed in minimal succinate medium containing a reduced amount of succinate (2 mM) and in the presence of 20 mM phenol, succinate, protocatechuate, *cis,cis*-muconate, catechol, glucose, fructose, glycerol, gluconate, 2-ketogluconate or mannitol. Growth was always lower for the mutant compared with the wild-type (results not shown), but in the case of gluconate, 2-ketogluconate and mannitol, almost no growth was observed for the PA4204 mutant (Fig. 6a, b, results shown for gluconate and mannitol). Interestingly, the large-colony pseudo-revertant could grow in these media (Fig. 6a, b). Complementation with the pBBR vector containing the wild-type PA4204 gene restored growth on gluconate, 2-ketogluconate, and mannitol (Fig. 6a, b), while the His-Ala substitution in PA4204 did not confer growth, again confirming the importance of this predicted active site residue (Fig. 6a, b).

Fig. 5. Expression and purification of the PA4204 protein cloned in the pET24 vector. (a) SDS-PAGE gel (12%) with overnight-induced crude extract of pET24 containing PA4204 (lane 2), and His-trap-bound protein fraction from overnight-induced crude extract (lane 3); lane 1, molecular mass markers. (b) HPLC separation of (S)-5-oxo-2-tetrahydrofurancarboxylic acid (left panel), and after addition of 16 µg purified protein (right panel). Below is the reaction described by Mochizuki (2006) for the *Pectobacterium (Erwinia) cypridii* homologue. (c) Gluconolactonase activity of the purified enzyme. The activity is measured as the drop in A_{405} of the pH indicator *p*-nitrophenol due to the acidification caused by the opening of the lactone ring of D-glucono-δ-lactone in the presence of 1.6 µg purified enzyme (●) or the enzyme boiled for 10 min (○). The rate of spontaneous hydrolysis is also shown (▲) as well as the inhibition caused by adding EDTA (0.01 M final) to the reaction mix containing the enzyme (△).

RT-PCR analysis of the phosphogluconolactonase gene (*pgl*)

As mentioned in the Introduction, *P. aeruginosa* has a *pgl* gene (PA3182) encoding a cytoplasmic phosphogluconolactonase. Because the product of PA4204 is a periplasmic gluconolactonase, we wanted to know whether the absence of this activity in the PA4204 mutant could affect the production of Pgl. As shown in Fig. 6(c), a clear reduction in the amount of cDNA was visible when the mRNA was

extracted from the small-colony PA4204 mutant, while an increased expression of *pgl* was apparent in the complemented strain and in the large-colony variant.

DISCUSSION

The PA4204 gene in *P. aeruginosa* is found upstream of the *mexGHI-opmD* operon, which encodes the components of an efflux pump known to have a close relationship with the

Fig. 6. Growth of wild-type *P. aeruginosa* (●), PA4204 mutant (○), PA4204 pseudorevertant (▲), PA4204 complemented with the wild-type gene (△), and PA4204 complemented with the His-Ala mutated gene (◆) in the presence of gluconate (a) or mannitol (b). (c) RT-PCR analysis of the expression of the PA3182 gene (*pgl*) encoding the cytoplasmic Pgl. Lanes: 1, wild-type; 2, PA4204 mutant (small-colony); 3, complemented PA4204 mutant; 4, large-colony variant of the PA4204 mutant. The bottom row shows the amplification of the *oprL* gene encoding the peptidoglycan-associated lipoprotein (PAL).

QS circuitry in this bacterium (Aendekerk *et al.*, 2002, 2005; Dietrich *et al.*, 2006; Bredenbruch *et al.*, 2006). Homologues of the PA4204 protein are present in the genomes of other fluorescent pseudomonads, such as *P. syringae*, but the *mexGHI-opmD* genes are found in *P. aeruginosa* alone. The genomic proximity to the pump genes prompted us to investigate the function of this gene and of the protein that it encodes. In this work, we show that the PA4204 mutant does not produce pyocyanin, which has been proposed recently to be a fourth signal molecule in *P. aeruginosa* (Dietrich *et al.*, 2006). However, the effect of PA4204 on QS may be indirect and due to a pleiotropic effect on the physiology of the bacterium, since this gene has never been found to be regulated by QS signal

molecules in the different genome-wide transcriptome analyses of the AHL- or PQS-mediated responses in *P. aeruginosa* (Schuster *et al.*, 2003; Wagner *et al.*, 2003; Hentzer *et al.*, 2003; Juhas *et al.*, 2004; Bredenbruch *et al.*, 2006). The exact function of PA4204 cannot yet be predicted unequivocally, but we confirmed here that it has a gluconolactonase activity. Although the protein contains a COG2706 domain corresponding to a CMLE, it also bears similarity to PGLs. However, PGLs are normally cytoplasmic, while the product of PA4204 is probably localized in the periplasm, a fact that we could confirm, since the signal peptide of PA4204 is processed when the gene is expressed in *E. coli* (Fig. 5). The protein encoded by PA4204 also has the same enzymic activity as the predicted

periplasmic enzyme described by Mochizuki (2003, 2006) in *Pectobacterium* (*Erwinia*) *cypripedii* 314B, with a lactonase activity for (S)-5-oxo-2-tetrahydrofuran-2-carboxylic acid, which converts this molecule to L- α -hydroxyglutaric acid. The substrate of the *Pectobacterium* (*Erwinia*) *cypripedii* lactonase, (S)-5-oxo-2-tetrahydrofuran-2-carboxylic acid, resembles 2-carboxy-2,5-dihydro-5-oxo-furan-2-acetate, better known as γ -carboxymuconolactone, one of the intermediates in the protocatechuate degradation pathway and the substrate of CMLE. In *P. aeruginosa*, the CMLE involved in the protocatechuate pathway is PcaB, which again is a cytoplasmic enzyme. There is, however, little similarity between PcaB and the PA4204 product. In fact, the putative PA4204-encoded CMLE is more closely related to the eukaryotic CMLEs represented by the *N. crassa* enzyme (Mazur *et al.*, 1994). We also demonstrated that the enzyme has a clear gluconolactonase

activity (Fig. 5c). An interesting observation is the absence of growth of the PA4204 mutant in minimal medium containing gluconate, 2-ketogluconate or mannitol. A *P. aeruginosa* *pgl* mutant (with defective cytoplasmic Pgl) has been described as failing to grow on mannitol and showing reduced growth on gluconate (Hager *et al.*, 2000). Here, we could demonstrate a similar phenotype for our PA4204 mutant, except that growth on gluconate was almost abolished. Furthermore, a clear decrease in expression of *pgl* results from the inactivation of PA4204, suggesting that the lactonization of gluconate in the periplasm reduces the amount of gluconate that can enter the ED pathway. Recently, a membrane sensor protein has been described in *P. aeruginosa*, which is needed for biofilm formation, production of the siderophore pyoverdine, and growth on mannitol and gluconate (Attila *et al.*, 2008). Interestingly, the PA4204 mutant also shows a strongly reduced capacity

Fig. 7. The glucose pathway in *P. aeruginosa* and possible function of PA4204. In the periplasm, glucose is transported via the Gct transporter into the cytoplasm, where the Gck glucokinase phosphorylates it into glucose 6-phosphate (G6P). G6P is oxidized by the glucose phosphate dehydrogenase Zwf to give δ -6-phosphogluconolactone (6PGL), which can isomerize into the toxic γ -6-phosphogluconolactone. The enzyme Pgl can convert the lactone into 6-phosphogluconate (6PGA), the central metabolite of the ED pathway. Gluconate can be oxidized in the periplasm by the membrane-bound Gcd dehydrogenase to give gluconate, which can be transported into the cytoplasm via the Gat transporter or oxidized further via another dehydrogenase, Gad, into 2-ketogluconate, which is transported into the cytoplasm via Kgt. According to the KEGG prediction, 2-ketogluconate can convert spontaneously to 2-dehydro-D-glucono-1,5-lactone. The function of the PA4204 enzyme in the periplasm could be to convert this lactone back into 2-ketogluconate. It is not known whether 2-dehydro-D-glucono-1,5-lactone is also transported into the cytoplasm.

to form biofilms (results not shown). We therefore propose a model that can explain the different phenotypic defects observed when PA4204 is inactivated (Fig. 7). The PA4204 enzyme is probably a periplasmic gluconolactonase active against either gluconolactone or a lactone compound (2-dehydro-D-glucono-1,5-lactone) that originates spontaneously from 2-ketogluconate, which itself is the product of the oxidation of gluconate by the membrane-bound gluconate dehydrogenase encoded by *gad* (PA2265). In *Pectobacterium* (*Erwinia*) *cypripedii*, the gluconate dehydrogenase is a three-component enzyme comprising a small subunit, the Gad flavoprotein and a *c*-type cytochrome (Yum *et al.*, 1997). In *P. aeruginosa*, the same genes (PA2264–PA2266) are found in the same order as in *Pectobacterium* (*Erwinia*) *cypripedii* (<http://www.pseudomonas.com>). The 2-ketogluconate molecule can isomerize spontaneously to form 2-dehydro-D-glucono-1,5-lactone (Kegg prediction). A possible function of the periplasmic PA4204 enzyme could be to convert this lactone (or gluconolactone) back into 2-ketogluconate (or gluconate), which can be transported to the cytoplasm. The fact that growth in the presence of glucose is not affected by the mutation of PA4204 is probably due to the fact that glucose can be transported to the cytoplasm, converted to glucose 6-phosphate and then to 6-phosphogluconate, and that the cytoplasmic Pgl suffices to ensure the hydrolysis of 6-phosphogluconolactone, thereby fuelling the ED pathway (Fig. 7). We hypothesize that in the absence of PA4204, gluconolactone and/or 2-dehydro-D-glucono-1,5-lactone accumulate in the periplasm and that these products are toxic to the cell, in agreement with the house-cleaning function suggested by Galperin *et al.* (2006). Alternatively, the compound could exert its toxicity in the cytoplasm, if it is transported to this cellular compartment. The mechanism by which gluconolactone or 2-dehydro-D-glucono-1,5-lactone inhibits the production of both AHLs and AQs is not clear, but both QS systems could be affected due to the perturbation in the general metabolism of the cell. Since the PA4204 periplasmic enzyme has a gluconolactonase activity, we propose to name this gene *ppgL* (periplasmic gluconolactonase).

More research will be required to further characterize the PpgL enzyme activity towards different lactones, including the predicted biologically relevant substrate 2-dehydro-D-glucono-1,5-lactone (which is not available commercially and needs to be chemically synthesized).

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