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PA2663 (PpyR) increases biofilm formation in *Pseudomonas aeruginosa* PAO1 through the *psl* operon and stimulates virulence and quorum-sensing phenotypes

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Abstract Previously, we identified the uncharacterized predicted membrane protein PA2663 of *Pseudomonas aeruginosa* PAO1 as a virulence factor using a poplar tree model; PA2663 was induced in the poplar rhizosphere and, upon inactivation, it caused 20-fold lower biofilm formation (Attila et al., Microb Biotechnol, 2008). Here, we confirmed that PA2663 is related to biofilm formation by restoring the wild-type phenotype by complementing the PA2663 mutation in *trans* and investigated the genetic basis of its influence on biofilm formation through whole-transcriptome and -phenotype studies. Upon inactivating PA2663 by transposon insertion, the *psl* operon that encodes a galactose- and mannose-rich exopolysaccharide was highly repressed (verified by RT-PCR). The inactivation of PA2663 also repressed 13 pyoverdine genes, which eliminated the production of the virulence factor pyoverdine in *P. aeruginosa*. The inactivation of PA2663 also affected other quorum-sensing-related phenotypes in that it repressed the *Pseudomonas* quinolone signal (PQS) genes, which abolished PQS production, and repressed *lasB*, which decreased elastase activity sevenfold. Genes were also induced for motility and attachment (PA0499, PA0993, PA2130, and PA4549) and for small molecule transport (PA0326, PA1541, PA1632, PA1971, PA2214, PA2215, PA2678, and PA3407). Phenotype arrays also showed that PA2663 represses growth on D-gluconic acid, D-mannitol, and *N*-phthaloyl-L-glutamic acid. Hence, the PA2663 gene product increases biofilm formation by increasing the *psl*-operon-derived exopolysaccharides and increases pyoverdine synthesis, PQS production, and elastase activity while reducing swarming and swimming motility. We speculate that PA2663 performs these myriad functions as a novel membrane sensor.

Keywords Biofilm · *Pseudomonas aeruginosa* · Pyoverdine · *psl* operon

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Introduction

Eighty percent of bacterial infections are caused by bacteria in biofilms (Costerton 2004), and *Pseudomonas aeruginosa* is notorious for biofilm formation (Sauer et al. 2004); hence, it is a model organism for biofilm formation (Caiazza et al. 2007). *P. aeruginosa* is a gram-negative, opportunistic bacterium that causes several acute and chronic infections (Filiatrault et al. 2006) in humans, animals, insects, nematodes, and plants (Lewenza et al. 2005). Its resistance to antibiotics and disinfectants makes it an important pathogen (Stover et al. 2000). With more than 150 predicted outer membrane proteins and four chemotaxis systems, *P. aeruginosa* is also an important bacterium for the transport and efflux of compounds (Stover et al. 2000).

The extracellular matrix of biofilms encloses bacterial cells and is comprised of polymeric substances such as nucleic acids, proteins, and polysaccharides (Friedman and

Kolter 2004); this biofilm matrix protects cells from host defenses (Fux et al. 2005). In *P. aeruginosa* PAO1, two operons, *pel* and *psl*, are important for biofilm formation. The *pel* operon affects biofilm maturation, and the *psl* operon affects biofilm initiation (Ma et al. 2006). The *psl* operon was identified by three different research groups (Friedman and Kolter 2004; Jackson et al. 2004; Matsukawa and Greenberg 2004), and the polysaccharide encoded by this operon is essential for preserving the biofilm structure of *P. aeruginosa* (Ma et al. 2006). The *psl* operon is not found in all *P. aeruginosa* strains; for example, it is absent in *P. aeruginosa* PA14 (Kuchma et al. 2007).

Other biofilm factors for *P. aeruginosa* include GacA, a response regulator (Parkins et al. 2001), which increases biofilm formation and antibiotic resistance (Parkins et al. 2001). Surface attachment is also important as *sadB* mutation impairs irreversible attachment of *P. aeruginosa* (Caiazza and O'Toole 2004). Another important biofilm determinant is iron, as it promotes both biofilm formation and pathogenicity because iron is essential for growth and is present at low concentrations (10^{-18} M) (Kaneko et al. 2007). *P. aeruginosa* has over 30 genes targeting iron (Kaneko et al. 2007) and is bound to iron-carriers (siderophores) to increase availability for bacterial cells (Michel et al. 2005). Pyoverdine (Merriman et al. 1995) and pyochelin (Michel et al. 2005) are the two siderophores produced by *P. aeruginosa*. In *P. aeruginosa*, pyoverdine controls three virulence factors: endotoxin A, elastase, and itself (Lamont et al. 2002); hence, pyoverdine is important for the pathogenicity of *P. aeruginosa*.

Another virulence factor of *P. aeruginosa* is *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone), a quorum-sensing signal that controls the production of pyocyanin, rhamnolipids, and elastase (Diggle et al. 2006). PQS regulation is intertwined with the other two quorum-sensing systems, Rhl and Las (Diggle et al. 2006).

Previously, we identified PA2663 as induced in a rhizosphere biofilm of *P. aeruginosa* formed on poplar trees vs a biofilm formed on inert glass wool (Attila et al. 2008). We showed that this mutant formed 20-fold and 11-fold less biofilm in Luria–Bertani (LB) and LB glu medium in 24 h, respectively, and that the inactivation of PA2663 reduced the virulence of *P. aeruginosa* toward barley (Attila et al. 2008). PA2663 is a putative membrane protein (Winsor et al. 2005). To investigate the genetic basis of this biofilm inhibition and reduction in virulence, DNA microarrays were used here to study the differential gene expression of *P. aeruginosa* PAO1 PA2663 vs the wild-type strain in biofilms. Phenotype arrays were also utilized to study the effect of the inactivation of PA2663 on metabolism. We found that PA2663 encodes a protein that influences the *psl* biofilm locus, pyoverdine formation, elastase activity, and PQS production; hence, we identified the first regulatory protein for the *psl* operon and another regulatory protein for quorum-sensing and virulence phenotypes.

Materials and methods

Bacterial strains, media, and growth conditions The strains and plasmids are listed in Table 1. *P. aeruginosa* was grown at 30°C. LB medium (Sambrook et al. 1989) was used for the crystal violet biofilm experiment, the glass wool biofilm DNA microarrays, PQS assays, elastase assays, rhamnolipid assays, and the swarming motility experiments. Minimal succinate medium (Ren et al. 2005b) was used to measure pyoverdine production, and minimal M9 medium (Rodriguez and Tait 1983) with 0.4% D-mannitol was used to evaluate growth on mannitol. Tetracycline (50 μg/ml) was used to select *P. aeruginosa* PAO1 transposon mutants, gentamycin (15 and 100 μg/ml) was used to select the *pslAB* mutant and *P. aeruginosa* PA14 transposon mutants, and chloramphenicol (200 μg/ml) was used to maintain plasmid-bearing strains.

Confirmation of PA2663 inactivation Four polymerase chain reactions (PCR) were used to verify the insertion of the IS*phoA*/hah transposon (4.83 kb) to deactivate PA2663. The IS*phoA*/hah transposon was inserted 51bp from the start codon of the PA2663 gene. With the PA2663-F and PA2663-R primers that amplify 914 bp of the PA2663 gene (Table 2), PCR was used to confirm the insertion of the transposon into the PA2663 mutant because these primers produced a 914-bp band for the PAO1 wild type but did not produce a band for the PA2663 mutant due to short elongation time (1 min). The HaH minus 138 and PA2663-R primers were used to verify the transposon insertion in the PA2663 mutant, but not in the PAO1 wild type by amplifying 484 bp of the PCR product corresponding to the 3′ end of the IS*phoA*/hah and a part of the PA2663 gene. The direction of the IS*phoA*/hah transposon was opposite to that of the PA2663 gene transcription. Similarly, the PA29650-F, 29650-R, GB3a, and R1 primers (Table 2) were used to confirm *MAR2xT7* transposon insertion and direction in the PA2663 gene homolog in *P. aeruginosa* PA14.

Construction of pMMB207-PA2663 PA2663 was amplified by Pfu DNA polymerase with the PA2663-forward and PA2663-reverse primers (Table 2) that create the *Eco*RI and *Hin*dIII restriction enzyme sites flanking 20 bp upstream of the start codon and 60 bp downstream of the stop codon of PA2663, respectively. The 360-bp PCR product was digested with *Eco*RI and *Hin*dIII and then ligated into

Table 1 *P. aeruginosa* PAO1 strains used in this study

Strains and plasmids	Genotype	Source
Strains		
P. aeruginosa PAO1 WT-UW	Wild-type strain from University of Washington	Jacobs et al. (2003)
P. aeruginosa PAO1 WT-DW	Wild-type strain from Daniel Wozniak	Jackson et al. (2004)
P. aeruginosa PAO1 WFPA60-DW	*ΔpslAB* mutant, Gen^R	Jackson et al. (2004)
P. aeruginosa PAO1 PA0996-UW	PA0996 Ω ISlacZ/hah, Tet^R; *pqsA*	Jacobs et al. (2003)
P. aeruginosa PAO1 PA2463-UBC	PA2463 Ω mini-*Tn*5-*luxCDABE,* Tet^R	Lewenza et al. (2005)
P. aeruginosa PAO1 PA2587-UW	PA2587 Ω IS*lacZ*/hah, Tet^R; *pqsH*	Jacobs et al. (2003)
P. aeruginosa PAO1 PA2662-UW	PA2662 Ω IS*phoA*/hah, Tet^R	Jacobs et al. (2003)
P. aeruginosa PAO1 PA2663-UW	PA2663 Ω ISphoA/hah, Tet^R	Jacobs et al. (2003)
P. aeruginosa PAO1 PA2664-UW	PA2664 Ω IS*lacZ*/hah, Tet^R	Jacobs et al. (2003)
P. aeruginosa PA14 WT	Wild-type PA14 strain	Liberati et al. (2006)
P. aeruginosa PA14_16250	*lasB* mutant, PA14_16250 Ω *MAR2xT7* Gen^R	Liberati et al. (2006)
P. aeruginosa PA14_19120	*rhlR* mutant, PA14_19120 Ω *MAR2xT7* Gen^R	Liberati et al. (2006)
P. aeruginosa PA14_29650	PA2663 homolog in PA14 strain, PA14_29650 Ω *MAR2xT7* Gen^R	Liberati et al. (2006)
P. aeruginosa PA14_33700	*pvdF* mutant, PA14_33700 Ω *MAR2xT7* Gen^R	Liberati et al. (2006)
Plasmids		
pMMB207	Chl^R, $lacI^q$	de Lorenzo et al. (1993)
pMMB207-PA2663	Chl^R, *ptac*::PA2663 in pMMB207	This study

Chl^R: chloramphenicol, *Gen*^{R}: gentamycin, *Tet*^{R}: tetracycline resistance, *UBC*: *P. aeruginosa* strains obtained from the University of British Columbia, *UW*: *P. aeruginosa* strains obtained from the University of Washington, *DW*: strains obtained from Prof. Daniel J. Wozniak

pMMB207, which encodes chloramphenicol resistance (de Lorenzo et al. 1993). The PA2663 gene and 118-bp upstream of the resulting plasmid, pMMB207-PA2663, were sequenced with primer Seq-PA2663 (Table 2) to confirm the presence of PA2663 using the ABI BigDye Terminator Cycle Sequencing Kit.

Crystal violet biofilm assay Biofilm formation in 96-well polystyrene plates was performed as indicated previously (Pratt and Kolter 1998) with 300 μl of crystal violet per well, and the absorbance was measured at 540 nm. Briefly, overnight cultures were inoculated with an initial turbidity of 0.05 at 600 nm at 30°C in LB and in LB supplemented with 0.2% (*w*/*v*) glucose medium for 7 and 24 h without shaking. The biofilm at the liquid–plastic interface and the total biofilm were measured by using crystal violet staining. The dye staining the biofilms was dissolved in 95% ethanol, and the total biofilm formation was measured at an optical density of 540 nm. Twelve replicates (6 wells from each of 2 independent cultures) were averaged to obtain each data point.

To complement the PA2663 mutation during biofilm formation, PA2663 expression was induced in *P. aeruginosa* PAO1 PA2663-UW via pMMB207-PA2663 by adding 0 to 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). The negative control was *P. aeruginosa* PAO1 PA2663-UW containing the empty pMMB207 plasmid.

Swimming and swarming motility assays Swimming motility experiments were performed as explained previously (Sperandio et al. 2002) by measuring halos on agar plates after 5 and 18 h. For swarming motility, LB supplemented with 0.5% (*w*/*v*) agar plates was used. Briefly, *P. aeruginosa* cultures were regrown to a turbidity of 1 at 600 nm,

Table 2 Primers used for PCR amplification, RT-PCR, and sequence verification in this study. Restriction enzyme sites italicized

Primer name	Nucleotide sequence (5′ to 3′)	Primer name	Nucleotide sequence (5′ to 3′)
PA2663-F	AGGAAGCTCAGTTGCAGCAC	PA2663-R	AGCAGGTCGAGCACTTCTTC
PA2663-forward	GCCCC*GAATTC*TTCGGAAAAAGGA GATCGAGA	PA2663-reverse	GCCCC*AAGCTT*AGGAAGAACGGG CGGAAG
29650-F	CACGAGCAGGTCGAGCACTT	29650-R	ATTTCGTGACGATGCCAGGC
GB3a	TACAGTTTACGAACCGAACAGGC	R1	ATCGACCCAAGTACCGCCAC
pslA-forward	GCAAGCTGGTGATCTTCTGGTTCA	*pslA*-reverse	TGTCGTGGTTGCGTACCAGGTATT
pslB-forward	TTCAAGATCAAGCGCATCGTGGTG	*pslB*-reverse	AGGTGGATTCGTTGGTGTTGAGGA
Seq-PA2663	CCGCTTCTGCGTTCTGATTT	HaH minus 138	CGGGTGCAGTAATATCGCCCT

and halos were measured at 24 and 48 h. Ten replicates (5 plates from each of 2 independent cultures) were used to evaluate motility in each strain.

Biofilm RNA isolation for DNA microarrays *P. aeruginosa* PAO1 and the PA2663 transposon mutant were grown overnight in LB (wild-type strain) and LB with 50 μg/ml tetracycline (PA2663 mutant). One milliliter of the overnight cultures (turbidity 7.5 at 600 nm) was inoculated into 250 ml of fresh LB medium with 10 g of glass wool (Corning Glass Works, Corning, NY, USA) (Ren et al. 2004). The cultures were incubated for 7 h at 30°C with shaking, then the glass wool was removed quickly and washed in 200 ml of 0°C 0.85% NaCl buffer for 30 s. The cells were removed from the glass wool by sonicating at 22 W (FS3 sonicator, Fisher Scientific, Pittsburg, PA, USA) for 2 min in 200 ml of 0.85% NaCl buffer at 0°C. The buffer was centrifuged at 10,000×*g* for 2 min at 4°C (J2-HS centrifuge, Beckman, Palo Alto, CA, USA) to obtain the cell pellets, and total RNA was isolated and checked as described previously (Ren et al. 2005a). To prevent mRNA decay, bacterial cultures were harvested quickly at 0°C, and the beadbeater vials for the bacterial cell pellets were kept at −80°C. All instruments to isolate RNA were wiped with RNaseZap (Ambion, Austin, TX, USA). In addition, the Qiagen Kit used to isolate RNA (#74104) contained Buffer RLT, which contains guanidine isothiocyanate and β-mercaptoethanol to inactivate RNases.

DNA microarrays The *P. aeruginosa* Genechip Genome Array (Affymetrix, P/N 900339), which contains 5,500 of the 5,570 open reading frames of *P. aeruginosa* (Whiteley et al. 2001), was used to analyze the *P. aeruginosa* transcriptome. cDNA synthesis, fragmentation, and hybridization were performed as described previously (Attila et al. 2008). The data were obtained by using the GeneChip operating software (Affymetrix). A gene was considered differentially expressed when the *p* value for comparing two microarrays was <0.05 (to assure that the change in gene expression was statistically significant, i.e., there are no more than 5% false positives). In addition, for each comparison of two genes, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*p* value<0.05), these genes were discarded. The intensities of polyadenosine RNA control were used to monitor the labeling process. Total signal intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The gene functions were obtained from the *Pseudomonas* Genome Database (http://v2.pseudomonas.com) (Winsor et al. 2005). The expression data have been deposited in the NCBI Gene Expression Omnibus (GSE9255) (Edgar et al. 2002).

Real-time polymerase chain reaction Real-time, reverse transcription polymerase chain reaction (RT-PCR) was performed to corroborate the microarray data as described previously (Lee et al. 2007). The primers were designed using PrimerQuest^{SM} online software (http://www.idtdna.com) (Table 2). RT-PCR was performed using an ABsolute™ MAX QRT-PCR SYBR Green Mixes Kit (ABgene House, UK) in a MyiQ™ Single Color Real-time PCR Detection System (BioRad Laboratories).

Pyoverdine production *P. aeruginosa* strains were grown in minimal succinate medium (Ren et al. 2005b), then the cells were diluted to a turbidity of 0.05 at 600 nm in fresh minimal succinate medium and were grown at 30°C. The experiment was performed as described previously (Ren et al. 2005b). Briefly, 1 ml of culture was centrifuged at 16,000×*g* for 1 min, and the supernatant was used to determine the pyoverdine concentration by measuring the absorption at 405 nm (Stintzi et al. 1998); these values were normalized by cell density.

PQS assay To assay PQS production, overnight cultures were diluted to a turbidity of 0.02 at 660 nm, and PQS was extracted as described previously (Gallagher et al. 2002; Pesci et al. 1999). Briefly, cells were grown for 24 h at 30°C, and 300 μl of the culture was extracted with acidified ethyl acetate (0.1 ml of glacial acetic acid in 1 l of ethyl acetate). The organic layer was transferred to a microcentrifuge tube and evaporated with a stream of nitrogen. Dried extracts were resuspended in 40 μl of 1:1 mixture of acidified ethyl acetate/acetonitrile. Synthetic PQS was used as a standard, and 10 μl of each extract was placed on Silica Gel 60 F_{254} plates (VWR, 5729-6). The solvent used for TLC was a 19:1 mixture of dichloromethane/methanol. The PQS levels were determined and photographed using a VersaDoc 3000 imaging system (BioRad, Hercules, CA, USA).

Elastase and rhamnolipid assays Elastase activity was assayed as described previously (Ohman et al. 1980). Briefly, overnight cultures were regrown to a turbidity of 1.5 at 600 nm. One milliliter of the bacterial cells was harvested and 100 μl of bacterial supernatant was incubated with 900 μl of Tris–HCl buffer and 20 mg of elastin-Congo Red (MP Biomedicals, 101637) for 3 h. The reaction was terminated by adding 350 μl of sodium phosphate buffer. The insoluble elastin-Congo Red was removed by centrifuging, and the absorbance of the supernatant was measured at 405 nm to determine the elastase activity.

Rhamnolipid activity was assayed as described previously (Wilhelm et al. 2007).

Phenotype microarray Phenotype PM1-8 microarray plates (12111, 12112, 12121, 12131, 12141, 12181, 12182, 12183) (Biolog, Hayward, CA, USA) were used to investigate 756 different phenotypes. Briefly, cells were removed from the BUG+B agar plates with a sterile swab and placed into IF-O base buffer overnight (30°C), and the cell turbidity at 600 nm was adjusted to 0.065. Bacterial inocula were prepared, and 100 µl of each cell suspension was inoculated on the plates.

Results

Differential gene expression in biofilms upon inactivating PA2663 To explore the mechanism by which PA2663 induces biofilm formation in *P. aeruginosa*, differential gene expression in biofilms was performed. The most striking changes in gene expression were found in the genes repressed upon inactivating PA2663, and these are shown in Table 3 (there were 71 genes repressed with a 50-fold cutoff and 49 genes induced with a 4-fold cutoff). Clearly, the repression of transcription was dominant; hence, PA2663 is an activator of gene expression.

Of the repressed genes, 15 genes of the *psl* operon that play an important role in biofilm formation in *P. aeruginosa* (Ma et al. 2006) were repressed consistently from 6- to 512-fold; for example, *pslA* and *pslB*, which encode proteins that are important in biofilm initiation (Jackson et al. 2004), were repressed by 79- and 512-fold, respectively. *pslD*, which encodes a secretion protein necessary for biofilm formation (Campisano et al. 2006), was also repressed by 194-fold change. Hence, the PA2663 protein is critical for the induction of the *psl* operon.

Thirteen pyoverdine genes were also repressed from 9- to 832-fold (Table 3). For example, *pvdA* was the most repressed pyoverdine gene (832-fold); PvdA (L-ornithine N^5-oxygenase) converts ornithine into N^5-formyl-N^5-hydroxyornithine (Lamont and Martin 2003; Visca et al. 1994). These data show that PA2663 is also critical for pyoverdine synthesis in *P. aeruginosa*.

Three anthranilate dioxygenase genes (*antABC*) were also repressed 91- to 416-fold upon inactivating PA2663. Anthranilate is a precursor of signal 2-heptyl-3-hydroxy-4-quinolone (PQS) (Urata et al. 2004) that controls the *rhl* quorum-sensing pathway, virulence, and biofilm formation (Diggle et al. 2006). Three arsenite-resistant (*arsB*, *arsC*, and *arsR*) genes were also repressed 6- to 64-fold; arsenite induces the oxidative stress response in *P. aeruginosa* (Parvatiyar et al. 2005). In addition, *bkdA1*, *bkdA2*, *bkdB*, and *lpdV*, which belong to a dehydrogenase operon (Winsor et al. 2005), were repressed in the biofilm formed by the PA2663 mutant by 74- to 832-fold. Previously, this gene cluster was induced in developing biofilms (Waite et al. 2006). Two more dehydrogenase genes, PA2265 (encodes gluconate dehydrogenase) (Winsor et al. 2005) and PA2290 (encodes glucose dehydrogenase) (Winsor et al. 2005), were also repressed by 79- and 158-fold, respectively.

Of the 49 induced genes (Table 4), 7 small molecule transport genes including a porin precursor gene *oprP* (Winsor et al. 2005) were induced by 4.3-fold. OprP controls the transport of phosphate ions in *P. aeruginosa* (Moraes et al. 2007). The *braZ* gene, which encodes a Na^+-coupled transporter of branched-chain amino acids, was induced fourfold. Two ATP-binding cassette transporter genes, *PA0326* and *PA2678* (Winsor et al. 2005), and four motility and attachment genes (PA0499, PA0993, PA2130, and PA4549) were also induced.

Verification of repression of pslAB by RT-PCR To corroborate the DNA microarrays, which indicated severe and consistent repression of the *psl* operon upon inactivation of PA2663 (e.g., *pslA* and *pslB* repressed 79- and 512-fold, respectively), RT-PCR was performed. We observed no amplification for these two genes in the RT-PCR reaction with PA2663 RNA while amplification was clear for the wild-type strain (template specificity was ensured by using melt-curve analysis). Therefore, these two genes are not expressed upon inactivating PA2663, which agrees with the DNA microarray analysis.

Biofilm formation of the PA2663 and pslAB mutants and complementation of PA2663 To corroborate the microarray studies and the link between reduced biofilm formation in the inactivated PA2663 mutant (20-fold biofilm reduction in 24 h in LB medium) (Attila et al. 2008) and repression of the *psl* operon, we investigated biofilm formation of the *pslAB* mutant (Jackson et al. 2004). The inactivation of PA2663 and *pslAB* decreased biofilm formation by 30-fold after 7 h in LB and LB glu media (Fig. 1). Hence, the *pslAB* and PA2663 mutations reduce biofilm formation to nearly the same extent in rich medium. In minimal glucose medium, only the *pslAB* mutant failed to form biofilm (35±8-fold less than the wild-type strain at 7 h). Biofilm reduction was not the result of a growth defect because the specific growth rate of PA2663 and the wild-type strain in LB medium are nearly identical (Attila et al. 2008), and the specific growth rate of the *pslAB* mutant was only 25% less than its wild-type strain.

To confirm that the decrease in biofilm formation is due to the mutation in PA2663, the mutant strain was

Table 3 Partial list of repressed genes for *P. aeruginosa* PAO1 PA2663 vs *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h

PA #	Gene name	Fold change	Descriptions
Pyoverdine genes			
PA2385	*pvdQ*	−13.9	Adaptation, protection, protein related to penicillin acylase
PA2386	*pvdA*	−831.7	Adaptation, protection, L-ornithine N^5-oxygenase
PA2393		−111.4	Central intermediary metabolism, probable dipeptide precursor
PA2394	*pvdN*	−97.0	Selenocysteine lyase, pyoverdine synthesis
PA2395	*pvdO*	−13.9	Pyoverdine synthesis
PA2396	*pvdF*	−36.8	Secreted factors, pyoverdine synthetase F, folate-dependent phosphoribosylglycinamide formyltransferase PurN
PA2397	*pvdE*	−42.2	Transport of small molecules, membrane protein, pyoverdine biosynthesis protein
PA2398	*fpvA*	−119.4	Transport of small molecules, ferripyoverdine receptor, outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid
PA2399	*pvdD*	−9.2	Secreted factors, pyoverdine synthetase D
PA2400	*pvdJ*	−104.0	Nonribosomal peptide synthetase modules and related proteins
PA2413	*pvdH*	−45.3	Adaptation, protection, L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, pyoverdine synthesis
PA2424	*pvdL*	−84.4	Adaptation, protection, nonribosomal peptide synthetase modules and related proteins, pyoverdine synthesis
PA2425	*pvdG*	−84.4	Predicted thioesterase involved in nonribosomal peptide biosynthesis, pyoverdine synthesis
PA2426	*pvdS*	−55.7	Transcriptional regulator, sigma factor PvdS
psl genes			
PA2231	*pslA*	−78.8	Probable glycosyl transferase, 69% similar to colonic acid UDP-glucose lipid carrier, WcaJ in *E. coli*
PA2232	*pslB*	−512.0	Probable phosphomannose isomerase/GDP-mannose pyrophosphorylase
PA2233	*pslC*	−111.4	Probable glycosyl transferase
PA2234	*pslD*	−194.0	Transport of small molecules, probable exopolysaccharide transporter
PA2235	*pslE*	−59.7	Uncharacterized protein involved in exopolysaccharide biosynthesis
PA2236	*pslF*	−36.8	Glycosyltransferase
PA2237	*pslG*	−222.9	Probable glycosyl hydrolase, β-xylosidase
PA2238	*pslH*	−104.0	Glycosyltransferase
PA2239	*pslI*	−97.0	Glycosyltransferase
PA2240	*pslJ*	−84.4	Part of *pslABCDEFGHIJKLMNO* cluster involved in biofilm development
PA2241	*pslK*	−5.7	Uncharacterized membrane protein, putative virulence factor
PA2242	*pslL*	−17.1	Fucose 4-*O*-acetylase and related acetyltransferases
PA2243	*pslM*	−17.1	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit
PA2244	*pslN*	−9.8	Topoisomerase IB
PA2245	*pslO*	−168.9	Hypothetical protein
PA2246	*bkdR*	−11.3	Transcriptional regulator
PA2247	*bkdA1*	−831.7	2-oxoisovalerate dehydrogenase
PA2248	*bkdA2*	−97.0	2-oxoisovalerate dehydrogenase
PA2249	*bkdB*	−73.5	Branched-chain alpha-keto acid dehydrogenase
PA2250	*lpdV*	−97.0	Amino acid biosynthesis and metabolism, energy metabolism, pyruvate 2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component
Biosynthesis of cofactors, prosthetic groups and carriers			
PA0996	*pqsA*	−3.0	Probable coenzyme A ligase, part of *pqsABCDE* operon
PA0997	*pqsB*	−4.3	Homologous to beta-keto-acyl carrier protein synthase, part of *pqsABCDE* operon
PA0998	*pqsC*	−3.5	Homologous to beta-keto-acyl carrier protein synthase, part of *pqsABCDE* operon
PA0999	*pqsD*	−2.8	3-oxoacyl synthase III, part of *pqsABCDE* operon
PA1000	*pqsE*	−2.1	Quinolone signal response protein, part of *pqsABCDE* operon
PA2587	*pqsH*	−2.6	Probable FAD-dependent monooxygenase
PA2512	*antA*	−415.9	Anthranilate dioxygenase large subunit

Table 3 (continued)

PA #	Gene name	Fold change	Descriptions
PA2513	*antB*	−168.9	Anthranilate dioxygenase small subunit
PA2514	*antC*	−90.5	Anthranilate dioxygenase reductase
Carbon compound metabolism			
PA2265		−78.8	Gluconate dehydrogenase, this family of proteins bind FAD as a cofactor
PA2290	*gcd*	−157.6	Energy metabolism, glucose dehydrogenase
Antibiotic resistance and susceptibility			
PA2491		−111.4	Probable oxidoreductase
PA2492	*mexT*	−6.1	Transcriptional regulator
PA2493	*mexE*	−6.5	Transport of small molecules, resistance-nodulation-cell division (RND) multidrug efflux membrane fusion protein MexE
PA2494	*mexF*	−55.7	Transport of small molecules, membrane protein, resistance-nodulation-cell division, multidrug efflux transporter MexF
PA2495	*oprN*	−4.0	Transport of small molecules, membrane protein, multidrug efflux outer membrane protein OprN precursor
PA2496		−59.7	Hypothetical protein
Amino acid biosynthesis and metabolism			
PA2443	*sdaA*	−45.3	L-serine dehydratase
PA2444	*glyA2*	−955.4	Serine hydroxymethyltransferase
PA2445	*gcvP2*	−137.2	Central intermediary metabolism, glycine cleavage system protein P2
PA2446	*gcvH2*	−84.4	Glycine cleavage system protein H2
Secreted factors, elastase			
PA3724	*lasB*	−4.0	Elastase lasB
Transport of small molecules			
PA2276		−59.7	Transcriptional regulator, AraC-type DNA-binding domain-containing proteins
PA2277	*arsR*	−5.7	Transcriptional regulator
PA2278	*arsB*	−6.5	Membrane proteins, transport of small molecules, Na^+/H^+ antiporter NhaD and related arsenite permeases
PA2279	*arsC*	−64.0	Adaptation, protection, protein phosphatase
Hypothetical, unclassified, unknown function			
PA2303		−52.0	Hypothetical protein, probable taurine catabolism dioxygenase
PA2304		−24.3	Hypothetical protein, probable taurine catabolism dioxygenase
PA2305		−48.5	Probable nonribosomal peptide synthetase
PA2306		−52.0	Membrane protein, putative threonine efflux protein
PA2330		−137.2	Hyothetical protein, Acyl-CoA dehydrogenases
PA2331		−238.9	Membrane protein
PA2363		−157.6	Hypothetical protein
PA2365		−207.9	Hypothetical protein, 71% similar to putative 19.5 kDa protein Eip20 in *Edwardsiella ictaluri*
PA2366		−1,024.0	Hypothetical protein, 76% similar to putative 19.5 kDa protein Eip55 in *Edwardsiella ictaluri*
PA2367		−724.1	Hypothetical protein, hemolysin-coregulated protein
PA2368		−238.9	Hypothetical protein
PA2369		−27.9	Membrane protein
PA2370		−119.4	Hypothetical protein
PA2371		−27.9	Translation, posttranslational modification, degradation, ATPases with chaperone activity
PA2372		−147.0	Hypothetical protein
PA2373		−39.4	Hypothetical protein, 46% similar to VgrG protein in *Escherichia coli*
PA2374		−52.0	Hypothetical protein
PA2375		−238.9	Membrane protein
PA2376		−5.7	Transcriptional regulator, response regulator containing a CheY-like receiver domain and an DNA-binding domain
PA2377		−4.9	Hypothetical protein, ABC-type Fe^{3+} transport system, periplasmic component

Table 3 (continued)

PA #	Gene name	Fold change	Descriptions
PA2378		−64.0	Probable aldehyde dehydrogenase, aerobic-type carbon monoxide dehydrogenase, CoxL/CutL homologs
PA2379		−9.2	Probable oxidoreductase
PA2380		−128.0	Hypothetical protein
PA2381		−362.0	Hypothetical protein
PA2382	*lldA*	−6.5	Energy metabolism, L-lactate dehydrogenase
PA2383		−78.8	Probable transcriptional regulator, 50% similar to positive regulator of *gcv* operon GcvA in *Escherichia coli*
PA2384		−111.4	Hypothetical protein
PA2403		−52.0	Membrane proteins, uncharacterized iron-regulated membrane protein
PA2404		−415.9	Membrane proteins
PA2405		−256.0	Type I export signal computationally predicted by LipoP v.1.0
PA2406		−18.4	Hypothetical protein
PA2407		−29.9	Motility and attachment, probable adhesion protein, ABC-type metal ion transport system
PA2408		−128.0	Transport of small molecules, probable ATP-binding component of ABC transporter
PA2409		−17.1	Membrane proteins, transport of small molecules, probable permease of ABC transporter
PA2410		−24.3	Hypothetical protein
PA2411		−73.5	Probable thioesterase, predicted thioesterase involved in nonribosomal peptide biosynthesis
PA2412		−512.0	Hypothetical protein
PA2414		−22.6	Carbon compound metabolism, L-sorbosone dehydrogenase
PA2415		−32.0	Membrane proteins
PA2416	*treA*	−13.0	Carbon compound catabolism, periplasmic trehalase precursor
PA2417		−18.4	Transcriptional regulator
PA2418		−147.0	Hypothetical protein, pirin-related protein
PA2419		−24.3	Probable hydrolase, amidases related to nicotinamidase
PA2420		−24.3	Membrane proteins, transport of small molecules, probable porin
PA2423		−111.4	Hypothetical protein
PA2433		−147.0	Hypothetical protein
PA2449		−52.0	Probable transcriptional regulator
PA2452		−274.4	Hypothetical protein
PA2453		−97.0	Hypothetical protein
PA2454		−39.4	2-polyprenyl-3-methyl-5-hydroxy-6-metoxy-1,4-benzoquinol methylase
PA2455		−32.0	Predicted acetyltransferase
PA2456		−168.9	Hypothetical protein
PA2501		−1,910.9	Membrane protein

Complete analysis deposited at the NCBI Gene Expression Omnibus (GSE9255).

complemented by measuring biofilm formation in LB and LB glu with pMMB207-PA2663. As expected, PA2663 expression increased biofilm formation of the PA2663 mutant in both media (6.4-fold in LB and 15.7-fold in LB glu) (Fig. 1).

To further investigate the relation of PA2663 and biofilm formation, we examined biofilm formation of the PA2663 mutant homolog in *P. aeruginosa* PA14 strain (*P. aeruginosa* PA14_29650), which does not possess the *psl* operon (Kuchma et al. 2007). As expected, the inactivation of the PA2663 homolog in *P. aeruginosa* PA14 does not inhibit biofilm formation in LB or LB glu (Fig. 1). This result confirms that PA2663 controls biofilm formation through the *psl* operon. We also examined the biofilm formation of the PA2662 and PA2664 mutants, which belong to the same operon as the PA2663 gene (Winsor et al. 2005). Mutation of these two genes did not alter biofilm formation (Fig. 1).

PA2663 decreases swimming and swarming motility Previously, we found that the PA2663 mutation increased 1.5-fold swimming motility after 18 h (Attila et al. 2008);

Table 4 Partial list of induced genes for *P. aeruginosa* PAO1 PA2663 vs *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h

PA #	Gene name	Fold change	Descriptions
Amino acid biosynthesis and metabolism			
PA0288	*gpuA*	4.0	3-guanidinopropionase, arginase/agmatinase/formimionoglutamate hydrolase, arginase family
PA1393	*cysC*	4.6	Central intermediary metabolism, nucleotide biosynthesis and metabolism, adenosine 5′-phosphosulfate kinase
Biosynthesis of cofactors, prosthetic groups and carriers			
PA4891	*ureE*	4.3	Urease accessory protein UreE
Carbon compound catabolism			
PA1384	*galE*	5.7	Central intermediary metabolism, Nucleotide biosynthesis and metabolism, UDP-glucose 4-epimerase
PA1983	*exaB*	5.7	Energy metabolism, cytochrome c550
PA2086		4.0	Probable epoxide hydrolase
PA2093		4.0	Probable sigma-70 factor, ECF
PA4150		4.0	Probable dehydrogenase E1 component
PA4151	*acoB*	6.1	Acetoin catabolism protein AcoB, pyruvate/2-oxoglutarate dehydrogenase complex
PA4152		3.7	Probable hydrolase
PA4153		4.9	2,3-butanediol dehydrogenase
PA5417	*soxD*	4.0	Sarcosine oxidase delta subunit
Cell wall, LPS, capsule			
PA1385		5.3	Probable glycosyltransferase
Motility and attachment			
PA0993	*cupC2*	4.0	Chaperones and heat shock proteins, P pilus assembly protein, chaperone PapD
PA2130	*cupA3*	4.0	Usher CupA3, P pilus assembly protein, porin PapC
PA4549	*fimT*	4.0	Type 4 fimbrial biogenesis protein FimT
Hypothetical, unclassified, unknown function			
PA0497		4.3	Hypothetical protein
PA0498		4.0	Hypothetical protein
PA0499		4.3	Motility and attachment, chaperones and heat shock proteins, P pilus assembly protein, chaperone PapD, 47% similar to filamentous hemagglutinin in *Bordetella pertussis*
PA0697		4.3	Hypothetical protein
PA0823		4.9	Hypothetical protein
PA0825		4.3	Hypothetical protein
PA0874		4.9	Hypothetical protein
PA0939		2.5	Rof, transcriptional antiterminator, 57% similar to *YaeO* (pleiotropic multicopy suppressor of Ts mutations; ribosome-associated; growth-rate regulated) in *E. coli*
PA0977		4.6	Hypothetical protein
PA0978		4.3	Related to phage, transposon, or plasmid, transposase and inactivated derivatives
PA0979		4.0	Related to phage, transposon, or plasmid, transposase and inactivated derivatives
PA1501		4.0	Hydroxypyruvate isomerase
PA1936		4.6	Hypothetical protein
PA1953		4.9	Predicted double-glycine peptidase
PA2209		4.0	Hypothetical protein
PA2935		7.0	Hypothetical protein
PA3412		4.3	Hypothetical, unclassified, unknown
PA3713	*spdH*	4.0	Spermidine dehydrogenase
PA4683		6.1	Hypothetical protein
PA4881		4.6	Hypothetical protein
PA5385		4.9	Hypothetical protein

Table 4 (continued)

PA #	Gene name	Fold change	Descriptions
Protein secretion, export apparatus			
PA1701		4.0	Conserved hypothetical protein in type III secretion
PA1856		4.3	Probable cytochrome oxidase subunit, 74% similar to the CytN gene product of *Azospirillum brasilense*
Transcriptional regulator and transport of small molecules			
PA0885		4.0	Probable C4-dicarboxylate transporter
Transport of small molecules			
PA0326		4.0	Probable ATP-binding component of ABC transporter
PA1541		4.6	Membrane protein, membrane transporters of cations and cationic drugs
PA1632	*kpdF*	4.9	KpdF protein
PA1971	*braZ*	4.0	Branched chain amino acid transporter BraZ
PA2214		4.3	Probable major facilitator superfamily (MFS) transporter, sugar phosphate permease
PA2215		4.6	Hypothetical protein, L-alanine-d,L-glutamate epimerase and related enzymes of enolase superfamily
PA2678		4.3	Membrane proteins, probable permease of ABC-2 transporter
PA3279	*oprP*	4.3	Phosphate-specific outer membrane porin OprP precursor
PA3407	*hasAp*	2.6	Heme acquisition protein

Complete analysis deposited at the NCBI Gene Expression Omnibus (GSE9255).

hence, we investigated the impact of this mutation on swarming. Compared to the wild-type strain, inactivating PA2663 increased swarming motility 2.1±0.3-fold after 48 h. The *pslAB* mutant also induced swimming motility 1.3±0.1-fold but repressed swarming motility 2.4±0.4-fold. As expected, the PA2663 mutant homolog in *P. aeruginosa* PA14 (which lacks *psl*) did not show any difference in swimming or swarming motility.

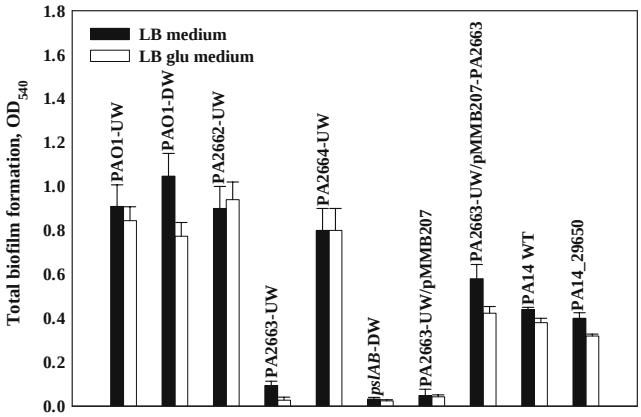

Fig. 1 Effect of the PA2663, PA2662, PA2664, and *pslAB* mutations on biofilm formation at 30°C after 7 h in 96-well plates with LB medium (*black bars*) and LB medium supplemented with 0.2 wt% glucose (*white bars*). At least two independent experiments were conducted (total of 12 wells), and *error bars* indicate 1 SD. *PAO1-UW* indicates the wild-type strain *P. aeruginosa* PAO1 from the University of Washington and *PAO1-DW* indicates the wild-type strain *P. aeruginosa* PAO1 from Prof. Daniel J. Wozniak

PA2663 stimulates pyoverdine production Because the DNA microarrays indicated that 13 pyoverdine genes were repressed, we measured pyoverdine synthesis. Upon inactivation of PA2663 in PAO1, pyoverdine production normalized by cell density (OD_{405}/OD_{600}) decreased up to 15-fold (Fig. 2). Therefore, as indicated by the whole transcriptome analysis, the inactivation of PA2663 eradicates pyoverdine synthesis. As a positive control, pyoverdine synthesis with the unrelated rhizosphere mutant *P. aeruginosa* PA2463-UBC was also checked, and no

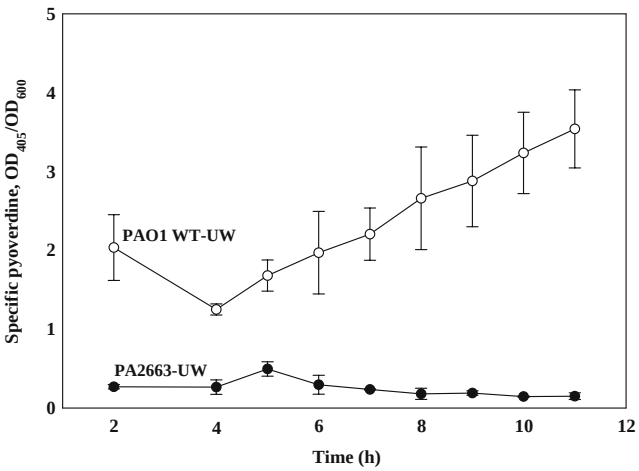

Fig. 2 Effect of the PA2663 mutation on cell density-normalized pyoverdine production. The absorbance at 405 nm was used to indicate pyoverdine production, and the absorbance at 600 nm was used to monitor cell density. *Error bars* indicate 1 SD

Table 5 Metabolic phenotype based on Biolog arrays upon inactivating PA2663 vs *P. aeruginosa* PAO1 WT at 24 h at 30°C

Compound	OD_{590} PA2663/OD_{590} wild type	Physiological role	Pathway
D-gluconic acid	0.01	Carbon source	Pentose phosphate pathway
D-mannitol	0.01	Carbon source	Fructose and mannose metabolism, ABC transporter
D-3-phospho-glyceric acid	0	Phosphorus and sulfur source	
D-psicose	7.24	Carbon source	
D-trehalose	0.09	Carbon source	Starch and sucrose metabolism
Glycine	0	Carbon source	Glycine, serine, and threonine metabolism
	0.12	Nitrogen source	
Glutathione	0.26	Phosphorus and sulfur source	Glutathione metabolism
L-cysteine	9.15	Nitrogen source	Cysteine metabolism, methionine metabolism, taurine and hypotaurine metabolism, thiamine metabolism, pantothenate and CoA biosynthesis, and sulfur metabolism
L-isoleucine	0	Carbon source	Valine, leucine, isoleucine biosynthesis and degradation
	0.33	Nitrogen source	
L-leucine	0.01	Carbon source	Valine, leucine, isoleucine biosynthesis and degradation
	0	Nitrogen source	
L-valine	0.16	Nitrogen source	Valine, leucine, isoleucine biosynthesis and degradation
L-methionine sulfone	0	Phosphorus and sulfur source	
L-pyroglutamic acid	16.0	Carbon source	
N-acetyl-D-glucosamine	7.8	Carbon source	
N-acetyl-D,l-methionine	0.13	Phosphorus and sulfur source	
N-phtaloyl-L-glutamic acid	0	Nitrogen source	
Parabanic acid	0	Nitrogen source	
S-methyl-L-cysteine	0.09	Phosphorus and sulfur source	
Tyramine	0.02	Carbon source	Tyrosine metabolism, alkaloid biosynthesis
	0	Nitrogen source	
Uracil	0.32	Nitrogen source	Pyrimidine metabolism, β-alanine metabolism, pantothenate and CoA biosynthesis
Uridine	0.35	Nitrogen source	Pyrimidine metabolism
Ala–Thr	0	Peptide nitrogen source	
Ala–Val	0	Peptide nitrogen source	
Gly–Gly	0	Peptide nitrogen source	
Gly–Gly–Gly	0	Peptide nitrogen source	
Gly–Gly–Ile	0	Peptide nitrogen source	
Gly–Gly–Leu	0	Peptide nitrogen source	
Gly–Phe–Phe	0	Peptide nitrogen source	
Gly–Val	0	Peptide nitrogen source	
Gly–Pro	0.44	Peptide nitrogen source	
Gly–Ser	0.18	Peptide nitrogen source	
Ile–Gly	0	Peptide nitrogen source	
Ile–Ser	0.15	Peptide nitrogen source	
Leu–Glu	0	Peptide nitrogen source	
Leu–Gly	0	Peptide nitrogen source	
Leu–Gly–Gly	0	Peptide nitrogen source	
Leu–Ile	0	Peptide nitrogen source	
Leu–Trp	0	Peptide nitrogen source	
Leu–Val	0.09	Peptide nitrogen source	
Phe–Gly–Gly	0	Peptide nitrogen source	
Pro–Val	0.06	Peptide nitrogen source	
Trp–Trp	0.07	Peptide nitrogen source	
Val–Leu	0	Peptide nitrogen source	
Val–Gly	0	Peptide nitrogen source	
Val–Tyr–Val	0.12	Peptide nitrogen source	

difference between the wild type and PA2463 was observed (data not shown). In addition, the negative control, a *pvdF* mutant, did not produce pyoverdine.

PA2663 stimulates PQS and elastase production The DNA microarrays showed that *pqsABCDEH* were repressed (Table 3). Therefore, we examined the PQS production in the PA2663 mutant. Inactivation of PA2663 abolished PQS production compared to the wild-type PAO1 strain. The PA0996-UW (*pqsA*) and PA2587-UW (*pqsH*) mutants were used as negative controls for PQS production. Furthermore, the DNA microarrays showed that *lasB* was repressed fourfold upon inactivating PA2663 (Table 3); therefore, we examined the elastase activity of the PA2663 mutant and found that the PA2663 mutant synthesized 7.0±0.7-fold less elastase activity compared to the wild-type PAO1 strain. A *P. aeruginosa* PA14 *lasB* mutant was used as the negative control for elastase activity. However, the PA2663 mutation did not alter rhamnolipid production. A *P. aeruginosa* PA14 *rhlR* mutant was used as a negative control for rhamnolipid production. Therefore, PA2663 is a positive effector for PQS and elastase.

PA2663 affects cell phenotypes Because the regulation of 129 genes was impacted by inactivating PA2663 as shown by the DNA microarrays (partial list shown in Tables 3 and 4), we explored the impact of inactivating PA2663 on 756 phenotypes using the phenotype arrays (Table 5). The phenotype arrays showed that inactivating PA2663 severely suppressed growth on many carbon sources (e.g., D-gluconic acid, D-mannitol, D-trehalose, glycine, L-leucine, L-isoleucine, and tyramine), severely suppressed growth with many nitrogen sources (e.g., glycine, L-isoleucine, L-leucine, L-valine, *N*-phthaloyl-L-glutamic acid, parabanic acid, tyramine, uracil, and uridine), and severely suppressed growth with phosphorus and sulfur sources (e.g., D-3-phosphoglyceric acid, glutathione, L-methionine sulfone, *N*-acetyl-D, L-methionine, and *S*-methyl-L-cysteine). The lack of growth of the PA2663 mutant on D-mannitol was confirmed using M9 mannitol medium; because both the wild-type PAO1 strain and the PAO1 *pslAB* mutant grew well in this medium, the PA2663 mutation prevents growth on D-mannitol.

Several of these compounds identified by the phenotype arrays are related to biofilm polysaccharide formation; for example, bacterial cellulose production in *Acetobacter xylinum* is enhanced by the inhibition of gluconic acid (Keshk and Sameshima 2006). The composition of the polysaccharide produced by the *psl* locus in *P. aeruginosa* has been investigated by two groups and found to consist mainly of mannose, rhamnose, and glucose, and contains *N*-acetyl quinovosamine, *N*-acetylglucosamine, and 3-deoxy-D-manno-octulosonic acid (Friedman and Kolter 2004) or consists chiefly of galactose, mannose, glucose, xylose, rhamnose, and *N*-acetylglucosamine (Ma et al. 2006); hence, mannose is important for this polysaccharide. Also, the polysaccharides of other gram-negative bacteria (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) are rich in mannose (Yokochi et al. 1990). Adding mannitol to the medium reduced both capsular polysaccharide and extracellular polysaccharide production in *Rhizobium leguminosarum* (Breedveld et al. 1991), and mannitol increases the extracellular polysaccharide secretion by *Thiobacillus versutus* mutants (Claassen et al. 1986); hence, the repression of the *psl* locus in the PA2663 mutant is probably related to the lack of growth on mannitol by this mutant, and the strain may normally convert mannitol into mannose for polysaccharide synthesis but this conversion is altered by the PA2663 mutation.

Other compounds identified by the phenotype arrays such as glutathione, glycine, isoleucine, leucine, valine (Cho et al. 2006), and trehalose are also biofilm-related. In biofilm cells, glutathione is involved in potassium efflux (Gillam et al. 2005); hence, the membrane protein PA2663 may affect potassium transport. Also, trehalose increases the exopolysaccharide production (Nicolaus et al. 2002).

The phenotype arrays also revealed that the PA2663 mutation induced growth on D-psicose, L-cysteine, L-pyroglutamic acid, and *N*-acetylglucosamine (Table 5). Because the polysaccharide matrix of *Actinobacillus pleuropneumoniae* (Izano et al. 2007) and *Bacillus anthracis* (Ezzell et al. 1990) consists of *N*-acetylglucosamine, and *N*-acetylglucosamine was found in the *psl* exopolysaccharides (Friedman and Kolter 2004; Ma et al. 2007), it appears that the inactivation of PA2663 alters the metabolism of this compound. Also, poly-*N*-acetylglucosamine is necessary for *Staphylococcus aureus* virulence (Kropec et al. 2005), so the changes in barley virulence of the PA2663 mutant may be related to the altered metabolism of this compound.

The inactivation of PA2663 also severely repressed the growth on many peptide mixtures as nitrogen sources (Table 5). These results imply that PA2663 mutation affects amino acid synthesis or transport.

Discussion

In this study, by investigating the changes that occur upon inactivating PA2663 (which encodes a putative transmembrane protein) in *P. aeruginosa* PAO1, we demonstrated that the PA2663 gene product increases the production of the virulence factor pyoverdine by activating 13 genes of the pyoverdine synthesis locus and increases biofilm formation by activating 15 genes of the *psl* locus. Along with the DNA microarrays, the other lines of evidence linking PA2663 to the *psl* operon include (1) RT-PCR showing the transcription of PA2663 is abolished upon

inactivation of PA2663, (2) the similarities in biofilm formation of the PA2663 mutant and the *pslAB* mutants, (3) the lack of a change in biofilm formation in *P. aeruginosa* PA14 when the homolog of PAO1 PA2663 is inactivated because PA14 lacks a *psl* operon, and (4) the lack growth of the PA2663 mutant on mannitol as found through the phenotype arrays (*psl*-encoded polysaccharide is mannose-rich and both mannose and mannitol are related to biofilm formation). The phenotype arrays also revealed that other building blocks for exopolysaccharides (e.g., D-gluconic acid) were no longer good carbon sources and that growth on another exopolysaccharide building block, *N*-acetylglucosamine, was increased; hence, the PA2663 protein appears to be related directly to exopolysaccharide synthesis via its stimulation of the *psl* operon and via sugar utilization, and PA2663 influences pyoverdine production. Therefore, we suggest *ppyR*, for *psl* and pyoverdine operon regulator, as the new name for this gene.

The inactivation of PA2663 repressed the transcription of 71 genes; hence, it appears that PA2663 (85 aa) is part of a signaling regulon as an activator. Sharing an operon with PA2663, the flanking genes PA2662 and PA2664 are both associated with the signal nitric oxide (NO). PA2664 is a flavohemoglobin protein that detoxifies NO to nitrate, and its gene is induced the most in response to NO (Firoved et al. 2004), and PA2662 is predicted to respond to NO (Winsor et al. 2005). NO is a reactive nitrogen intermediate that reduces bacterial attachment, enhances motility, and induces dispersal in *P. aeruginosa* (Barraud et al. 2006). Therefore, it appears that PA2663 is also part of the NO regulon because it reduces biofilm formation, and its role needs to be elucidated. Most transporters have six or more transmembrane regions, and many bacterial membrane signals have two transmembrane regions (von Heijne 2006). Because PA2663 has only two transmembrane regions, 14-IGHGLLAAGLALLVAGVIAAYFL-36 and 45-LVFSHALVILGPTLL KIGYVMRL-67 as predicted by SOSUI (Hirokawa et al. 1998), PA2663 is unlikely to be a transporter and is probably a novel type of membrane sensor that regulates exopolysaccharide and pyoverdine production perhaps via NO.

It is also evident that PA2663 works through the PQS and *las* quorum-sensing systems. PQS genes (*pqsABCDEH*) were repressed in our microarray analysis (Table 3), and the inactivation of PA2663 reduced PQS production (note that the transcription of *antABC* was also controlled). In addition, *lasB* was repressed (Table 3) and elastase activity was controlled by PA2663. Pyoverdine is also controlled by the Las system (Stintzi et al. 1998), and its production was markedly reduced. Hence, along with the stimulation of biofilm formation, PA2663 controls quorum-sensing phenotypes including pyoverdine production. Because the knowledge of specific *P. aeruginosa* genes that are required for biofilm formation may lead to novel therapeutic agents (Junker and Clardy 2007), it is interesting that another protein (encoded by PA2663) that enhances biofilm formation and controls virulence factors and quorum-sensing phenotypes has been identified.

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