ORIGINAL ARTICLE

Characterization of a novel gene related to antibiotic susceptibility in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a human pathogen with increased intrinsic resistance to a large number of antibiotics used in clinical therapy. Therefore, understanding the mechanisms of resistance and developing therapy alternatives for *P. aeruginosa* are of profound importance. Previous work from our laboratory demonstrated that several mutants have isolated with altered expression of the *phzA1B1C1D1E1F1G1* (*phzA1*) operon in the presence of sub-inhibitory concentrations (SICs) of tetracycline (TET). The present study investigates the roles of the *PA0011* gene in mediating *phzA1* expression at SIC of TET. The *PA0011* gene encodes 2-OH-lauroyltransferase by controlling the synthesis of the cell envelope and the outer membrane. We found that the *PA0011* mutant strain was susceptible to several different antibiotics and environmental stresses. Complementation in the *PA0011* mutant restored these phenotypes to wild-type levels. In addition, expression of the *PA0011* gene, as monitored through a luciferase reporter, is increased at SICs of antibiotics. Indeed, the expression of the *PA0011* gene increased about threefold in *pqsR* and *pqsH* mutants compared with the wild-type PAO1. However, the *PA0011* gene negatively regulates the quorum sensing (QS) system. Taken together, these data suggest that *PA0011* is involved in susceptibility to antimicrobial agents in *P. aeruginosa*, and that its susceptibility effect maybe partly dependent on increased QS expression.

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INTRODUCTION

Pseudomonas aeruginosa is naturally more resistant to antibacterial agents than many other Gram-negative species of bacteria, including *Escherichia coli*.¹ The growing problem of antibiotic resistance among bacterial pathogens and the escalating difficulty in finding new antibiotics are driving the search for a new approach to antibacterial chemotherapy.² There are many resistance mechanisms to antibiotics that exist in nature and in *P. aeruginosa*. A number of non-essential bacterial proteins may potentially contribute to intrinsic resistance. The susceptibilities of bacteria to antibiotics depends on many factors, including the structure and the composition of the cell envelope, the presence of inactivating enzymes and the availability of efflux pumps.^{3–5} All these mechanisms are potentiated by the relative impermeability of the outer membrane to both hydrophilic and hydrophobic drugs.⁶ This outer membrane property in *P. aeruginosa* is mostly dependent on the structural heterogeneity of porin OprF, as only a small proportion of this protein forms through the lipid bilayer.⁷ The smaller antibiotics, such as erythromycin and novobiocin, cannot efficiently cross the cell envelope via the porin uptake pathway.¹ Lipopolysaccharides (LPS) are the major components on the outer surface of the outer membranes of Gram-negative bacteria, including *P. aeruginosa* and *E. coli*, and form a barrier around the cell.^{8,9} Some low-MW compounds can penetrate the outer membrane

via porins, but larger, potentially harmful agents, including many antibiotics, are excluded.¹⁰ As with antimicrobials, extensive use has led to increasing quinolone resistance among bacterial pathogens. Resistance to fluoroquinolones by *P. aeruginosa* is often mediated by mutations in target enzymes or upregulation in efflux pump expression, coupled with intrinsically low outer membrane permeability.^{11,12}

Quorum sensing (QS) is a global regulatory system controlling population behavior, and has a fundamental role in bacterial pathogenicity.¹³ There are three intertwined QS systems in *P. aeruginosa*: the acyl-homoserine lactone (HSL) mediated *las* and *rhl* systems, and the 2-alkyl-4(1 *H*)-quinolone (AHQ) signal-based system.^{14–17} The *las* and *rhl* systems consist of the transcriptional activators LasR and RhlR, and the signal synthases LasI and RhlI, respectively. The signals in the *las* and *rhl* systems are N-(3-oxododecanoyl)-HSL (3-oxo-C₁₂-HSL) and N-butyryl-HSL (C₄-HSL), respectively.¹⁶ The other cell–cell signaling system in *P. aeruginosa* functions through the AHQ signal-based system.¹⁴ The major AHQ signals include 2-heptyl-3-hydroxy-4-quinolone, the *Pseudomonas* quinolone signal (PQS) and 2-heptyl-4-quinolone.¹⁴ These systems control the production of numerous virulence factors.^{18,19} On top of the QS systems, there is a complex regulatory network that controls the QS systems both at a transcriptional level and at a post-transcriptional level.¹⁶ Recently, a study demonstrated that QS system regulation was mediated by

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sub-inhibitory concentrations (SICs) of antibiotics and was a potential target for anti-infective therapy.²⁰

Previously, we observed that SICs of antibiotics, including tetracycline (TET), activated phenazine production in *P. aeruginosa*, and we identified 32 transposon mutants that influenced expression of the phenazine synthesis gene (*phzA1*). Among them was the PA0011 mutant (LH02), in which *phzA1* expression was increased about eightfold by TET compared with that in the wild-type PAO1.²¹ Here, we report that the PA0011 mutant is not only susceptible to an array of antibiotics but also involved in the regulation of the QS system.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and derivatives were routinely grown at 37 °C on LB (Luria-Bertani) agar plates or in LB broth with shaking at 200 r.p.m. For *E. coli*, gentamicin (Gm) was used at 15 µg ml⁻¹, TET at 10 µg ml⁻¹, kanamycin at 50 µg ml⁻¹ and ampicillin at 100 µg ml⁻¹. For *P. aeruginosa*, Gm was used at 50 µg ml⁻¹ in LB and 150 µg ml⁻¹ in *Pseudomonas* isolation agar, TET at 75 µg ml⁻¹ in LB and 200 µg ml⁻¹ in *Pseudomonas* isolation agar, carbenicillin was used at 500 µg ml⁻¹ in *Pseudomonas* isolation agar and trimethoprim at 300 µg ml⁻¹.

DNA analysis and manipulation

Restriction enzymes and DNA polymerase or T4 ligase were purchased from Fermentas or Takara and were used under the conditions recommended by the manufacturers. Plasmid DNA was isolated using a plasmid kit (Biotek, Beijing, China). Treatment of DNA with enzymes, subcloning of DNA and transformation of plasmids into *E. coli* and *P. aeruginosa* were carried out using standard methods. Where required, DNA fragments were isolated from agarose gels using a Fermentas gel extraction kit. Standard methods were used for the preparation of electro-competent cells and for plasmid electroporation into *E. coli* and *P. aeruginosa*.²² Nucleotide sequences were determined using a 377A DNA sequencer (Sangon, Shanghai). DNA sequences were compared with the BLASTN program using information from a *Pseudomonas* database (<http://www.pseudomonas.com>).

Gene-replacement mutagenesis

For gene replacement, the previously described *sacB*-based strategy was employed.²³ The suicide plasmid, pKD0011K, for gene replacement was constructed by inserting the *Bam*HI-digested Gm^r-*lacZ* cassette derived from pZ1918-*lacZ*²⁴ between two PCR fragments of PA0011 cloned into pEX18Ap. The 533 bp upstream fragment of PA0011 was amplified using the primers, NH1 (5'-CGGCTGCAGTGCCTTCGGATGTCCAC-3') containing a *Pst*I site (underlined), and NH2 (5'-GCCGGATCCGCTTGGCGTAGGAGCAAT-3') containing a *Bam*HI site (underlined). The 537 bp downstream region of PA0011 was generated using primers, NH3 (5'-CGCGGATCCGCCCAAGCT

Table 1 Strains and plasmids used and constructed in this study

Strain or plasmid	Relevant feature	Source or reference
<i>Strains</i>		
<i>P. aeruginosa</i>		
PAO1	<i>Pseudomonas aeruginosa</i> wild type	This lab
LH02	PA0011 transposon mutant of PAO1, Gm ^r	This study
PAO(Δ0011)	PA0011 replacement mutant of PAO1, PA0011:: Gm ^r	This study
PAO(Δ0011)C	PAO(Δ0011) complemented strain	This study
Δ <i>pqsR</i>	<i>pqsR</i> replacement mutant of PAO1, Gm ^r	This study
Δ <i>pqsH</i>	<i>pqsH</i> replacement mutant of PAO1, Gm ^r	This study
<i>E. coli</i>		
	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>)80Δ <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i>	
DH5α	<i>endA1</i> <i>araD139</i> Δ (<i>ara</i> <i>leu</i>)7697 <i>galU</i> <i>galK</i> Δ- <i>rpsL</i> <i>nupG</i>	Invitrogen
Sm10	Mobilizing strain, RP4 integrated in the chromosome; Kn ^r	47
<i>Plasmid</i>		
pEX18Ap	oriT ⁺ <i>sacB</i> ⁺ gene replacement vector with multiple-cloning site from pUC18, Ap ^r	23
pZ1918 <i>lacZ</i> Gm	Source plasmid of Gm ^r cassette	24
pUCP28T	Broad-host-range vector	48
pMS402	Expression reporter plasmid carrying the promoterless <i>luxCDABE</i> ; Kn ^r Tmp ^r	26
pKD-PA0011	pMS402 containing PA0011 promoter region	This study
pKD0011K	PA0011 knock out plasmid pEX18Ap with 533 bp upstream region, Gm ^r - <i>lacZ</i> fragment from pZ1918Gm- <i>lacZ</i> and 537 bp downstream of PA0011 cloned between <i>Pst</i> I and <i>Eco</i> RI; Ap ^r (Cb ^r), Gm ^r	This study
pKD0011C	Complementation plasmid, pUCP28T with a 1161 PCR fragment covering the entire PA0011 gene and 125 bp upstream and 65 bp downstream regions between <i>Hind</i> III and <i>Pst</i> I; Tmp ^r	This study
pKD- <i>lasI</i>	pMS402 containing <i>lasI</i> promoter region	49
pKD- <i>rhII</i>	pMS402 containing <i>rhII</i> promoter region	49
pKD- <i>lasR</i>	pMS402 containing <i>lasR</i> promoter region	49
pKD- <i>rhIR</i>	pMS402 containing <i>rhIR</i> promoter region	49

GAAGGCAG-3') containing a *Bam*HI site and NH4 (5'-GCGGAATTCGGACCTGCGGAGGAG-3') containing an *Eco*RI site.

The plasmid, pKD0011K, was transferred to PAO1 using a tri-parental mating procedure employing the helper vector pRK2013.²⁵ Briefly, overnight cultures (25 ml of each) of *E. coli* strains containing plasmids pKD0011K and pRK2013, respectively, and *P. aeruginosa* PAO1 were centrifuged and the pellets resuspended in phosphate-buffered saline, then mixed with equal portions and spotted onto an LB agar plate. Following incubation overnight at 37 °C, bacteria were removed from the agar surface and resuspended in 1 ml of phosphate-buffered saline. Appropriate dilutions were plated onto *Pseudomonas* isolation agar containing Gm at 150 µg ml⁻¹. Subsequently, strains that had undergone a second crossover event were selected for by plating on LB supplemented with 5% sucrose supplemented with Gm. The PA0011 gene replacement mutant, designated PAO(Δ0011), was verified by assessing Gm resistance, carbenicillin sensitivity and PCR analysis.

Complementation of the PA0011 mutant

For the complementation experiments, PCR amplification was used to obtain a 1161-bp fragment of PA0011 containing its own promoter using the primers, 5'-GCG AAGCTTCATCTGCTACGCCTTCAT-3' and 5'-GCGCTGCAGCATAGTACCCGGATATG-3'. Restriction sites are shown as underlined, and were incorporated into the PCR primers. The fragment was digested with *Hind*III and *Pst*I, and the generated fragment was subsequently ligated into a *Hind*III-*Pst*I-digested broad-host-range vector, pUCP28T, to yield pKD0011C. The resultant pKD0011C was transformed into *P. aeruginosa* as described previously.²²

Construction of PA0011-*luxCDABE*

The plasmid pMS402 carrying a promoterless *luxCDABE* reporter gene cluster was used to construct promoter-*luxCDABE* reporter fusions of the PA0011 operon as reported previously.^{21,26} The promoter regions of PA0011 were PCR amplified using high-fidelity *Pfx* DNA polymerase and primers synthesized according to the PAO1 genome data.²⁷ The primer pairs used were: 5'-CGCCTCGAGACCATCTGCTACGCCTT-3' and 5'-CGCGGATCCAGGTTGATCCGCACTAC-3'. The restriction sites, shown as underlined, were incorporated into the PCR primers. The promoter regions were cloned into the *Xho*I-*Bam*HI site upstream of the *lux* genes in pMS402. The constructs were transformed into PAO1 by electroporation. DNA manipulation, PCR and transformation were performed following general procedures. Cloned promoter sequences were confirmed by DNA sequencing.

MIC determination

To determine the MICs of antibiotics, overnight cultures were diluted such that the OD at 600 nm (*A*₆₀₀) was 0.2, and cultivated for an additional 2 h before use as an inoculant. The cultures were inoculated into parallel wells of a 96-well plate with a transparent bottom. A 5-µl volume of the fresh cultures was seeded into the wells containing 195 µl medium plus antibiotics in the first well, and the OD of the cultures adjusted to 0.07 by performing 1:1 serial dilutions in a 96-well microtiter plate. The OD of culture at 595 nm was measured every 30 min for 24 h in the Victor³ Multilabel Plate Reader (Perkin-Elmer, Waltham, MA, USA).

Stress assays

The susceptibilities of wild-type PAO1 and the PA0011 mutant strain to osmotic stress and heat shock were determined as previously described.^{1,28} In the heat shock experiments, cells were grown overnight in LB at 37 °C and with aeration, then washed twice and diluted in M9 minimal medium to a density of ~7000 colony-forming units per ml (CFU ml⁻¹). One milliliter of the diluted culture was placed in a pre-warmed tube at 50 °C, and the viability was determined by taking periodic aliquots and plating them directly onto LB agar plates. In order to measure sensitivity to osmotic stress, the strains were grown in 35 ml of LB medium in 250 ml flasks to an *A*₆₀₀ of 0.3. At this point, the cultures were divided into two parts: one part was incubated with NaCl at a final concentration of 1.5 M; and the other was incubated without NaCl.

Luminescence screening assays

Using these *lux*-based reporters, gene expression in liquid cultures was measured as counts per second (c.p.s.) of light production in a Victor³ Multilabel Plate Reader (Perkin-Elmer) or Synergy 2 (Biotek, Winooski, VT, USA), as previously described by our group. Overnight cultures of the reporter strains were diluted to an *A*₆₀₀ of 0.2 and cultivated for an additional 2 h before use. The cultures were inoculated into parallel wells of a black 96-well plate with a transparent bottom. A 5 µl volume of the fresh cultures was inoculated into the wells containing a total volume of 95 µl medium plus other components, and the *A*₆₀₀ value in the wells was adjusted to around 0.07. A 60 µl volume of filter-sterilized mineral oil was added to prevent evaporation during the assay. Promoter activities were measured every 30 min for 24 h. Bacterial growth was monitored at the same time by measuring the OD at 595 nm in a Victor³ Multilabel Plate Reader. Expression on solid medium was carried out by plating the reporter strains onto soft top LB agar and imaging in a LAS300 Imaging System (Fuji Corp., Tokyo, Japan).

RESULTS

Antimicrobial susceptibility testing

Our previous work has reported that expression of the *phzA1* operon in the LH02 strain is increased about eightfold compared with that in the wild-type PAO1 at 1/16 MIC of TET.²¹ However, the growth rate of LH02 was inhibited when cultured in LB medium with 1/4 MIC of TET (Figure 1a). To address whether PA0011 serves as a possible target for antimicrobial agents, insertional inactivation of the PA0011 gene was performed in *P. aeruginosa* PAO1. The MICs of the PA0011 mutant and wild-type PAO1 are summarized in Table 2. The data showed that carbenicillin, TET, ciprofloxacin, chloramphenicol, polymyxin, rifampin and erythromycin MICs for the PA0011 mutant were

Figure 1 Growth profile and phenotype of wild-type PAO1 and the PA0011 mutant. (a) The growth of wild-type PAO1 and PAO1(Δ0011) in LB medium with or without 1.25 µg ml⁻¹ TET. Wild-type PAO1 (○) and PAO1(Δ0011) (■) cultured in LB. Wild-type PAO1 (△) and PAO1(Δ0011) (▼) in LB with 1.25 µg ml⁻¹ TET. Values are means ± standard deviations of triplicate experiments. The growth rate of mutant was considered to be statistically significant (Student's *t*-test, *P* < 0.005) compared with the wild-type PAO1. (b) Light microscopic images of crystal violet-stained cells at a magnification ×100 after 16 h culture on LB agar plate. The average diameter of PAO (1.65 ± 0.43 µm) and PAO1(Δ0011) (1.89 ± 0.57 µm).

at least twofold decreased from that for wild-type PAO1. As expected, the MICs of the complement PA0011 mutant was restored to wild-type PAO1 levels. Indeed, microscopic examination of cell size after staining with crystal violet revealed the diameter of the PA0011 mutant ($1.89 \pm 0.57 \mu\text{m}$) was larger compared with wild-type PAO1 ($1.65 \pm 0.43 \mu\text{m}$) (Figure 1b). These morphological patterns were similar to those observed when *P. aeruginosa* are exposed to SICs of cell wall-active antibiotics, such as β -lactams.²⁹

Response of PA0011 to environmental stress

Our data showed that the PA0011 mutant was susceptible to numerous antibiotics. This made us wonder whether the PA0011 mutant was subject to other environmental stresses. The abilities of the PA0011 mutant and the wild-type PAO1 to resist drastic changes in temperature and osmolarity were investigated. Stationary-phase cultures of both strains were exposed to a sudden shift in temperature, 37–50 °C. After 2–20 min at 50 °C, lethality in the PA0011 mutant cultures was higher than that in the parent cultures (Figure 2a). After 20 min, the lethality of the mutant strain was about 20 times higher than the wild type. Exposure of the PA0011 gene rendered the cells more sensitive to both temperature and osmotic variations than its wild-type counterpart (Figure 2b). When the cells were subjected to an increase in osmotic pressure caused by the addition of a high concentration of salt, the PA0011 mutant was more sensitive (data not shown). These results indicate that the PA0011 gene responds to environmental stresses, such as temperature and osmotic variations.

Table 2 Antibiotic susceptibilities for the parent (PAO1), mutant and complement strains

Strain	MIC ($\mu\text{g ml}^{-1}$)						
	CIP ^a	Tet	Cm	Poly	Rif	Cb	Em
<i>P. aeruginosa</i>	0.4 ± 0.1	10 ± 2	100 ± 10	3 ± 1	25 ± 5	125 ± 15	200 ± 20
LH02	0.1 ± 0	4 ± 1	30 ± 5	1.5 ± 0.5	10 ± 2	40 ± 5	90 ± 10
PAO(Δ 0011)	0.1 ± 0	3 ± 1	25 ± 5	1.5 ± 0.5	10 ± 2	40 ± 5	90 ± 10
PAO(Δ 0011)C	0.3 ± 0.1	8 ± 1	100 ± 10	3 ± 1	25 ± 5	125 ± 10	180 ± 20

^aAbbreviations: Cb, carbenicillin; CIP, ciprofloxacin; Cm, chloramphenicol; Em, erythromycin; Poly, polymyxin; Rif, rifampin; Tet, tetracycline.

Expression of the PA0011::luxCDABE was activated by SICs of antibiotics

We have shown that the PA0011 gene was related to antibiotics and environmental stresses. On the basis of these observations, we speculated that the activation of the PA0011 gene may be regulated by antibiotics. To test this hypothesis, the promoter–reporter fusion plasmid of PA0011::luxCDABE was constructed and its activation investigated in the presence of antibiotics.

A disk-fusion method was initially used to visualize the response to antibiotics. The reporter strain carrying the PA0011 promoter was spread onto agar in a petri dish. The tested antibiotics were spotted onto filter discs laid on top of the agar. A positive response was observed as a change in light emission from the reporter strain beyond the zone of inhibition. The white light intensity reflected the promoter activity activated by these antibiotics, with the edge of the zone demonstrating inhibition ability. As shown in Figure 3a, the PA0011 promoter is activated by TET, streptomycin, azitromycin, erythromycin and ciprofloxacin. It is slightly activated by Gm, chloramphenicol, spectinomycin but no activation was observed with polymyxin and rifampicin.

Further analysis of promoter regulation using SICs of antibiotics was carried out in multiwell plates and expression was measured as light production (c.p.s.) on a Multilabel Plate Reader. We used TET and erythromycin as the example to illustrate the effect at SICs of antibiotics on the activation of PA0011 in LB broth. The data showed that the activation of PA0011 was upregulated by about twofold at SICs of TET and erythromycin (Figure 3b). The activities observed were similar to that on the LB agar plate assay.

Interaction between PA0011 and PQS

Recent research has demonstrated that PQS does not follow the classical QS signal tenet, but is instead trafficked within the *P. aeruginosa* population via membrane vesicles.³⁰ membrane vesicles are a bilayer with an outer leaflet of LPS³¹ and an inner leaflet of phospholipids. LPS is composed of lipid A, core oligosaccharides and the polysaccharide O-antigen.³² Mazumder and colleagues³⁰ have shown that PQS interacts with the LPS component of the outer membrane to initiate membrane vesicle formation in *P. aeruginosa*. On the basis of these observations, we hypothesized that PQS may affect the expression of the PA0011 gene. To determine the relationship between PA0011 and the PQS system, the activation of the promoter–reporter fusion pKD-PA0011 was tested in wild-type PAO1 and *pqsR*,

Figure 2 Susceptibility to temperature or osmotic stresses for the wild-type PAO1 and PA0011 mutant. (a) To assay cell survival of PAO1 (◆), PAO1(Δ 0011)C (▲) and PAO1(Δ 0011) (■) strains after exposure to heat shock at 50 °C. Viability was expressed as a percentage of the number of CFU at time zero. (b) To assay cell survival of PAO1 (◆), PAO1(Δ 0011)C (▲) and PAO1(Δ 0011) (■) strains after exposure to 1.5 M NaCl. Cell viability was determined after 2, 4 and 6 h. One hundred percent viability corresponded to the number of CFU present immediately after resuspension of the cells in 1.5 M NaCl. Values are means $\pm$ standard deviations of triplicate heat and osmotic shock experiments.

Figure 3 Activation of PA0011 in LB conditions with different antibiotics. (a, b) The PA0011 promoter was activated by antibiotics on solid plates. The left panel is a standard photograph of the plate showing the zones of inhibition. On the right is an image taken in the dark showing the specific activation of the PA0011 promoter by antibiotics. The white light intensity reflects the promoter activity, with the edge of the zone reflecting inhibition ability. (c) Expression profiles of the PA0011-*luxCDABE* transcriptional fusion reporter in a 96-well plate assay. The plasmid, pKD-PA0011, was transformed into wild-type PAO1 and the strain cultured in LB (white bars) or LB with 1/16 (black bars) and 1/32 MIC (slash bars) TET and Em. Values are means $\pm$ standard deviations of triplicate experiments. Azi, azitromycin; Cb, carbenicillin; CIP, ciprofloxacin; Cm, chloramphenicol; c.p.s., counts per second; Em, erythromycin; Poly, polymyxin; Sm, streptomycin; SPc, spectinomycin.

Figure 4 Expression profiles of PA0011 in *pqsR* (a) and *pqsH* (b) mutant strains. *P. aeruginosa* wild type (◆) and mutant strains (■) containing the promoter-reporter fusion plasmid pKD-PA0011 cultured in LB medium. The corresponding growth curves are shown by dashed lines. The expression of pKD-PA0011 in mutants was considered to be statistically significant (Student's *t*-test, $P < 0.005$) compared with the wild-type PAO1.

pqsH mutants, which are not capable of PQS production. The data showed the expression of PA0011 increased about twofold in *pqsR* and *pqsH* mutants compared with the wild-type PAO1 (Figure 4).

Transcriptional expression of the QS genes in the PA0011 mutant

Previous work has shown that the QS system is associated with multidrug resistance and biofilm formation in bacteria.³³ Murakami *et al.*³⁴ has also reported that the alternative sigma factor, RpoS, has a role in antibiotic tolerance in *P. aeruginosa*. Our data showed that the PA0011 gene was negatively regulated by the PQS system. Indeed, the PQS system is correlated to the *las* and *rhl* systems.³⁵ Based on these observations, we analyzed the expression of QS-genes in PA0011 mutant. The promoter-reporter fusions pKD-*lasI*, pKD-*lasR*, pKD-

rhlI and pKD-*rhlR* were transformed into the PA0011 mutant and their expression was screened with a Multilabel Plate Reader Victor³ 1420. The results are shown in Figure 5. The activation of *lasI*, *lasR* and *rhlI* increased at least twofold at stationary phase in PA0011 mutant. Indeed, the activation of the QS-promoter at SICs of TET in the PA0011 mutant compared with wild type, exhibited a notable increase in QS expression (Figure 5), indicating PA0011 gene negatively regulates QS system. However, no differences of *pqsR* and *pqsH* were observed between them (data not shown).

DISCUSSION

In this study, we reported the isolation and characterization of a transposon mutant from a *P. aeruginosa* mutagenesis library.²¹

Figure 5 Expression profiles of the QS genes in wild-type PAO1 and the PA0011 mutant. The expression profiles of the promoter–reporter fusions of *lasR* (a), *lasI* (b), *rhlR* (c) and *rhlI* (d) were measured every 30 min. The cultures were placed in an aerobic jar and incubated at 37 °C. The gray and white bars represented the expression of the promoter in wild-type PAO1 cultured in LB medium with or without 0.625 µg ml⁻¹ TET, respectively. The striped and black bars represent the expression of the promoter in PA0011 mutant cultured in LB medium with or without 0.625 µg ml⁻¹ TET, respectively. Values are means ± s.d. of triplicate experiments.

The *PA0011* gene encodes 2-OH-lauroyltransferase, and we find the PA0011 mutant is more susceptible to a large variety of antibacterial agents than its parental strain PAO1 (Figure 1a, Table 1). The drug susceptibility of the PAO1(Δ0011) strain was also accompanied by regulation of QS systems and reduced resistance to osmotic and heat shock stress. Indeed, the activation of PA0011 was induced by numerous antibiotics (Figure 3). These data presented suggest that the PA0011 mutant, depending on the growth phase and the nutritional balance in its environment, allows for different triggers of survival under stressful conditions, such as antibiotic addition.

Dotsch *et al.*³⁶ has shown that 40 mutants were confirmed to be hyper-susceptible to several different antibiotics in *P. aeruginosa*. As we know, disrupting the *mexA* and *mexB* genes, which encode the major intrinsic multidrug MexAB-oprM efflux pump in *P. aeruginosa*,³⁷ exhibited the most pronounced overall susceptibility. Insertions into the genes involved in cell envelope biogenesis resulted in hyper-susceptibility, including *wzz* gene, which is important for determining the length of the O-antigen side chain attached to LPS and influential in serum resistance. Indeed, genes encoding putative lipoprotein and outer membrane proteins, such as OprF, OmpH and PA2800, were shown to be susceptible. In the present study, the PA0011 mutant was constructed and shown to be susceptible to a large variety of antibiotics compared with the wild-type PAO1 (Table 2). The PA0011 mutant strain was also sensitive to 1.5 M NaCl and heat shock (Figure 2). The *PA0011* gene encodes 2-OH-lauroyltransferase that is required for 3-deoxy-D-manno-octulosonic acid (Kdo) addition in lipid A biosynthesis and can rapidly incorporate one laurate residue into (Kdo)₂-lipid IVA.³⁸ Moreover, outer membrane proteins are key molecules with regard to the interface between the cell and its environment, and these proteins have been suggested to influence the intrinsic resistance to antibiotics. Using a proteomic approach, a *Vibrio vulnificus ompH* mutant showed enhanced sensitivity to SDS

and polymyxin B.³⁹ Therefore, all the altered phenotypes in the PA0011 mutant may be due to deficiencies of lauroyltransferase influence during synthesis of the cell membrane. The detailed mechanism needs to be investigated in future.

Recent antimicrobial research has focused on induction of the SOS response by antibiotics and the role that SOS response has in resistance development.⁴⁰ In addition to the well-characterized role of inhibitory concentrations, SICs of antibiotics may also be critical in this context. In our previous work, a transposon mutagenesis library was used to examine the effects at SICs of TET on virulence gene expression in *P. aeruginosa*.²¹ Moreover, this was expended here with the addition of TET and concentrations of other antibiotics. Interestingly, the expression of QS-controlled genes, such as *phzA1* and *rhlA* (data not shown) and *Rhl*-QS genes in PA0011 mutant, were up-regulated in condition with TET (Figure 5). Skindersoe *et al.*⁴¹ showed that TET had little effect on QS, but that low concentrations of azithromycin, ceftazidime and ciprofloxacin have QS inhibitory activity in *P. aeruginosa*. Indeed, previous studies have shown that SICs of ceftazidime, tobramycin and azithromycin reduce the QS signals in *P. aeruginosa*.^{42,43} In contrast, our results indicated that SICs of TET significantly activated the expression of QS-controlled genes in PA0011 mutant. Accordingly, we assumed that PA0011 may act as a receptor to influence the expression of virulence factors in the presence of SICs antibiotics.

QS in *P. aeruginosa* consists of a complex network and there are at least three QS systems; that is, *las*, *rhl* and AHQ.¹⁶ A hierarchy within the *N*-acyl-HSL-based QS system is believed to exist in which the *las* system controls the *rhl* system. PQS, as one of the signals of AHQ, is intricately connected to the AHL systems.¹⁵ The *rhl* and *las* systems, respectively, exert negative and positive regulation on PQS, whereas PQS positively influences the *rhl* system. PqsR(MvfR) is the response regulator in the PQS system.⁴⁴ The results have shown that the

expression of *las* and *rhl* was affected in the PA0011 mutant (Figure 5). Indeed, the PA0011 promoter activity was increased in the *pqsR* and *pqsH* mutant strains (Figure 4). Therefore, it is possible that the PQS system regulates the *rhl/las* systems via the PA0011 gene.

Increasingly, a global concern has emerged that infections controlled by interfering with QS systems can be effective, and QS has been used as a target when people discover new antibiotics.^{45,46} Effective means to attenuate or inhibit pathogenicity involve in controlling or disturbing expression of QS systems.²⁰ Characteristics of the newly identified mutant, PA0011, show that it is sensitive to an array of antibiotics, and contributes to QS systems expression. Therefore, it may enable researchers to elucidate new antibiotic targets in *P. aeruginosa*.

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