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Pleiotropic effects of temperature-regulated 2-OH-lauroytransferase (PA0011) on *Pseudomonas aeruginosa* antibiotic resistance, virulence and type III secretion system

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ABSTRACT

Pseudomonas aeruginosa is an important human pathogen which adapts to changing environment, such as temperature variations and entering host by regulating their gene expression. Here, we report that gene PA0011 in *P. aeruginosa* PAO1, which encodes a 2-OH-lauroytransferase participating in lipid A biosynthesis, is involved in carbapenem resistance and virulence in a temperature-regulated manner in PAO1. The expression of PA0011 was higher at an environment temperature (21 °C) than that at a body temperature (37 °C). The inactivation of PA0011 rendered increased antibiotic susceptibility and decreased virulence both *in vivo* and *in vitro*. The impaired integrity and the decreased stability of the outer membrane were the cause of the increased susceptibility of PAO1(Δ0011) to carbapenem and many other common antibiotics. The reduced endotoxic activity of lipopolysaccharide (LPS) contributed to the decreased virulence both at 21 °C and 37 °C in PAO1 (Δ0011). In addition, we have found that PA0011 repressed the expression of TTSS virulence factors both at transcriptional and translational levels, similar to the effect of O antigen of LPS but unlike any effect of its homologue reported in other bacteria. The effect of PA0011 on resistance to many antibiotics including carbapenem and virulence in *P. aeruginosa* makes it a target for novel antimicrobial therapies.

1. Introduction

Pseudomonas aeruginosa (PA) is an important opportunistic Gram-negative pathogen leading to nosocomial infections [1,2]. It is the major cause of morbidity and mortality among compromised patients such as HIV patients, burn victims and cystic fibrosis (CF) patients [3].

The infection by *P. aeruginosa* is difficult to eradicate due to both high intrinsic and acquired resistance to a wide variety of antibiotics including carbapenems, the first line of treatment against the infection by *P. aeruginosa* [4]. The emergence and spread of strains with resistance to kinds of antibiotics, especially to carbapenems, post a challenge to the success of antibiotics therapy. Decreased expression of outer membrane porins [5], activity of inducible β-lactamases including metallon β-lactamase and cephalosporinase [6], and multidrug efflux systems [7] etc. have been identified as the mechanism of carbapenem resistance in *P. aeruginosa*.

The development of antimicrobial resistance is essential to enable pathogenic bacteria to overcome antimicrobial therapies and to survive in competitive environments [8]. It is crucial to investigate the molecular mechanisms of antibiotic resistance as well as pathogenesis in order to optimize antibiotic chemotherapy and find new drug targets against *P. aeruginosa* infections.

To further investigate the mechanisms of *P. aeruginosa* drug resistance, a *P. aeruginosa* transposon insertion library was constructed and screened for the genes involved in the resistance to imipenem (Imp), meropenem (Mem) and biapenem (Bip) in PAO1. PA0011 was identified from three transposon insertion mutants with the obviously increased susceptibility to the three

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carbapenems compared with the wild type PAO1.

The PA0011 product in *P. aeruginosa* has been annotated as a secondary lipid A acyltransferases (*htrB*) based on a previous report [9] and its sequence similarity to the lpxL in *E. coli* [10]. Lipid A, acts as the hydrophobic anchor of LPS in the outer surface of the outer membrane [11–13], and consists of a D-glucosamine disaccharide phosphated at positions 1 and 4 and acylated at several different positions by first as well as second acylation in different species [10]. PA0011 has been found to affect the expression of *phzA1B1C1D1E1F1G1* (*phzA1*) operon in the presence of sub-inhibitory concentration (SIC) of tetracycline in our previous report [14].

In this study, the involvement of PA0011 in carbapenem resistance and pathogenicity has been investigated, as well as the mechanism of such involvement. Our results indicated that PA0011 plays an important role in both carbapenem resistance and pathogenicity in a temperature-dependent manner. Its function in maintaining outer membrane integrity and stability has been investigated together with its influence on the endotoxicity of LPS and motility. PA0011 was also found to influence the expression of type III secretion system (TTSS) both in transcriptional and translational levels negatively. The results suggested that PA0011 is involved in the antibiotics resistance and virulence through its influence on LPS, and influences TTSS system negatively. The results confirming the function of PA0011 as a lipid A lauroyltransferase is presented, and the relation of lipid A or LPS and TTSS has been discussed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and *Escherichia coli* DH10B strains were generally grown at 21 °C or 37 °C on *Pseudomonas* isolate agar (PIA) plates, Luria–Bertani (LB) agar plates, or in LB liquid with shaking at 200 rpm. Kanamycin (Kan, 50 μg/mL), gentamicin (Gm, 15 μg/mL), ampicillin (Amp, 100 μg/mL) and tetracycline (Tc, 15 μg/mL) were added to LB when necessary. Trimethoprim (Tmp, 300 μg/mL), Gm (150 μg/mL), Tc (300 μg/mL) were added to PIA. All antibiotics used were purchased from Amresco (Solon, USA). SDS and EDTA, purchased from Sigma–Aldrich, were prepared as filter sterilized stock solutions of 10% SDS and 50 mM EDTA (pH 7.5) respectively.

2.2. Construction of replacement mutant strains

The knockout mutant PAO1(Δ0011) was constructed by allelic exchange with the pEX18 sucrose counter-selection system [23]. Briefly, the 1.4 kb upstream fragment of PA0011 was amplified using a forward primer (5′-TAgaattcAGCGGATGTCGTGGACCAG-3′) and a reverse primer (5′-CATggatccGAGCAATAGAAGTGGTTGAG-3′) containing *Eco*RI and *Bam*HI restriction sites underlined. The 1.3 kb downstream region of *PA0011* was generated with the forward primer (5′-TAggatccCTGAAGGCAGTGGACGAGT-3′) and the reverse primer (5′-TCCaagcttGTTGCCGAGGATGG-3′) containing *Bam*HI and *Hin*dIII site. The upstream and downstream fragments of PA0011 were digested and then ligated into pEX18Tc to gain the plasmid pEX18Tc-PA0011. The *Bam*HI-digested Sm^r-Ω cassette derived from pHP45Ω was inserted into the two PCR fragments of PA0011 of pEX18Tc-PA0011 to generate plasmid pEX18Tc-PA0011-Ω.

The PA0011 knockout mutant was obtained by means of triparental mating as reported previously [24]. Briefly, 30 mL of overnight cultures of the donor strain *E. coli* containing the plasmid pEX18Tc-0011-Ω, the recipient PAO1 and the helper strain containing pRK2013 were collected and resuspended in PBS. The bacteria were mixed in a ratio of 2:1:2 and then spotted onto LB agar plates. After culturing at 37 °C for 12–16 h, the bacteria were scraped and resuspended in 1 ml LB. The diluted suspensions were spread on PIA plates containing streptomycin at 150 μg/mL and 10% sucrose. The resultant PA0011 knockout mutant was verified by PCR and designated as PAO1(Δ0011).

2.3. Complementation of PA0011 knockout mutant

To complement the PA0011 knockout mutant, PA0011 gene together with its promoter region was PCR amplified and integrated into the *attB* site on the chromosome by means of the mini-CTX system [25]. The primers used in the PCR amplification were the forward primer (5′-CGTAgaattcATGCGTTCGGATGTTC-3′) and the reverse primer (5′-GTATggtaccGCGAGTTCACGGATTA-3′), and the underlined are the *Eco*RI and *Kpn*I sites. The PCR product was ligated into miniCTX-*lacZ* to generate plasmid pC0011 which was then transferred into *E. coli* SM10-λpir. Transfer of pC0011 into the PAO1(Δ0011) genome was carried out by biparental mating. Integrants were selected on PIA containing 300 μg/mL Tc. The integrase on pC0011 promoted the integration of the vector into the attB site on the *P. aeruginosa* genome. The plasmid portion of pC0011 was resolved from the chromosome by means of the Flp recombinase encoded by pFLP2 [23]. The resultant strain was designated as PAO1(Δ0011)C.

2.4. Construction of gene expression detecting systems

The plasmid pMS402 carrying a promoterless *luxCDABE* reporter gene cluster was used to construct promoter-luxCDABE reporter fusions as reported previously [26]. *PA0011* fragments containing the promoter regions of 0.3 kb was PCR amplified using the forward primer (5′-TATctcgagCAGGACTGCGACCGTTACG-3′) and the reverse primer (5′-CTCggatccCACATCAGCCAGCCTATG-3′) with *Xho*I and *Bam*HI sites underlined. The purified PCR product was cloned into the upstream of the lux genes on pMS402 to obtain pKD-*PA0011* reporter fusions. pKD-*PA0011* was introduced into PAO1 by electroporation. The luciferase gene *luxCDABE* allows the level of PA0011 expression to be monitored as light production and image was taken in a LAS3000 imaging system (Fuji Corp.).

Similarly, the promoter-luxCDABE reporter fusions of *exsC*, *exsD*, *exoS*, *exoY* and *exoT* was also constructed as described previously [27]. These reporter fusions were transformed into PAO1(Δ0011) and PAO1 by electroporation.

The expression of these genes was monitored by light production. Overnight cultures of PAO1(Δ0011) containing one of the above reporter fusions were diluted 1:10 and cultivated for two additional hours before used as inoculants. 5 μL of the fresh culture was inoculated to 95 μL LB broth with Tmp in the wells of a black clear-bottom 96-well plate (Costar 3614). 40 μL of filter-sterilized mineral oil (sigma) was added to the wells to prevent evaporation during the assay. PAO1 harboring the same reporter fusions were used as the controls. Both luminescence (counts per second, c.p.s.) and absorbance (595 nm) were measured every 30 min for 24 h in a Victor 3 Multilabel Counter (PerkinElmer, USA).

2.5. RNA extraction and real-time quantitative PCR (RT-qPCR)

RNA was extracted using RNAprep Pure Cell/Bacteria Kit (TIANGEN). Then 3 μL RNA sample was reverse transcribed to cDNA using 1st Strand cDNA Synthesis Kit (Takara). Real-time qPCR was performed using SuperReal PerMix Plus (SYBR Green/probe) (TIANGEN) and primers specific for *exoT* (Forward: 5′-TCTCAGCAGAACCCGTCTTTCGTGGCTGAG-3′, reverse: 5′-AGCATCATCTGCTTGATCTCGGCGGCAGAG-3′), *exoS* (Forward: 5′-

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Genotype or phenotype	References
Strains		
E. coli		
DH10B	F *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ*139*Δ(*ara-leu*)*7697 galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
SM10-*λpir*	*Thi-1 thr leu tonA lacY supE recA::RP4-2-Tc:Mu*, Kan^R, *λpir*	[15]
P. aeruginosa		
PAO1	Wild type	[16]
PAO1(Δ0011)	PA0011 replacement mutant with a Ω-Sm cassette inserted, Sm^R	This study
PAO1(Δ0011)C	PAO1(Δ0011) complemented strain, PAO1(Δ0011) *attB*::PA0011	This study
PAO1(Δ0011)O	PA0011 overexpression strain containing plasmid pAK-0011 in PAO(Δ0011), Cb^R	This study
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB*, Tc^R	[17]
pHP45Ω	The pHP45 vector included a circlc and the Ω fragment in linear form; Sm/Spe^R	[18]
pRK2013	Broad-host-range helper vector; Tra^+, Kan^R	[19]
pAK1900	*E. coli-P. aeruginosa* shuttle cloning vector carrying plac upstream of MCS, Amp^R, Cb^R	[20]
pAK-0011	pAK1900 with a 1.0 kb PCR fragment of PA0011 gene	This study
mini-CTX-*lacZ*	Integration plasmid, Tc^R	[21]
pFLP2	2.6-Kb *Bam*HI–*Sph*I fragment from pALB2 ligated between the same sites of pPS908, Amp^R	[17]
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE*, Kan^R,Tmp^R	[22]
pKD-*exsC*	pMS402 containing *exsC* promoter region; Kan^R,Tmp^R	This lab
pKD-*exsD*	pMS402 containing *exsD* promoter region; Kan^R,Tmp^R	This lab
pKD-*exoT*	pMS402 containing *esoT* promoter region; Kan^R,Tmp^R	This lab
pKD-*exoY*	pMS402 containing *exoY* promoter region; Kan^R,Tmp^R	This lab
pKD-*exoS*	pMS402 containing *exoS* promoter region; Kan^R,Tmp^R	This lab
pKD-*PA0011*	pMS402 containing PA0011 promoter region; Kan^R,Tmp^R	This study

AAGCGGTAGAGAGCGAGGTC-3′, reverse: 5′-GCCCTCTTCCACTG CGTTGA-3′), *exsD* (Forward; 5-GCCGGGTTACGCATCGAGCACTTT-3, Reverse; 5-CGCCGCGCAGAGGAGAATC-3), PA0011 (Forward: 5′-TCGCTGAGATACATC CGCGA-3′, reverse: 5′-GAGTGGAAGGTGC-GACGCG-3′) and normalized to the housekeeping gene *proC* (Forward: 5′-CAGGCCGGGCAGTTGCTGTC-3′, reverse: 5′-GGTCAGGCGCGAGGCTGTCT -3′).

2.6. Antibiotics susceptibility test

The susceptibilities of *P. aeruginosa* PAO1, PAO1(Δ0011) and PAO1(Δ0011)C strains to the antimicrobial agents (Imp, Mem, Bip) were analyzed by detecting minimum inhibitory concentrations (MICs) and disk diffusion measurement. Serial dilutions were made to determine the MIC of the antibiotics by measuring the growth of the strains in 96-well plates (Costar, USA). MICs were recorded as the lowest concentration of antibiotic inhibiting visible growth after 18 h of incubation at 37 °C. For disk diffusion, 10 μL of the 50 μg/mL antibiotic was spotted on filter disks of 6 mm diameter which were placed on LB agar plates spread with 40 μL $OD_{600} \approx 0.5$ bacterial cells. The plates were incubated at 37 °C for 12 h and at 21 °C for 20 h. The diameters of the clear zones around the antimicrobial agents were observed.

2.7. Bacteria quantification

The stability assay of the outer membrane described by Malinverni, J.C was used [28]. Overnight cultures in LB broth were transferred into a 96-well plate in duplicates and 10-fold dilutions were made 6–7 times with fresh LB broth. 5 μL dilutions from the wells were transferred to LB agar plates with or without SDS-EDTA (0.5% SDS, 1.4 mM or 1.6 mM EDTA). Colonies were counted after 12 h incubation at 37 °C and 20 h at 21 °C.

2.8. In vivo virulence assays and quantitative bacteriology

A Chinese cabbage (*Brassica pekinensis*) infection model based on the lettuce infection model by Rahme et al. [29] and a fruit fly (Drosophila melanogaster Canton S) feeding model [30] were used to compare the virulence of PAO1(Δ0011) and the wild type as well as PAO1(Δ0011)C.

Bacteria were grown overnight at 21 °C or 37 °C in LB. Cells were collected and washed in 10 mM $MgSO_4$. After diluted to 10^7 CFU/mL, 10 μL of the dilution was inoculated to the stems of Chinese cabbage with a micropipette. The stems were washed with 0.1% H_2O_2 before inoculation. Inoculated stems were placed in a 15 cm diameter Petri dishes containing a filter impregnated with 10 mM $MgSO_4$. The dishes were kept in a growth chamber of 21 °C and 37 °C respectively and monitored daily for 6 days or 3 days. Same midrib of the cabbage was inoculated with the PAO1 or PAO1(Δ0011)C for comparison.

The Drosophila melanogaster feeding assay was carried out as described previously [30]. The wild type PAO1, PAO1(Δ0011) and PAO1(Δ0011)C were cultivated overnight at 21 °C and 37 °C in LB medium and the cultures were adjusted to an $OD_{600} = 2.0$ with LB. The pellets from 1.5 mL adjusted cultures were suspended in 100 μL 5% sucrose. The suspensions were spotted onto a sterile filter (Whatman GF/A 21 mm) placed on the surface of 5% sucrose agar in the wells of a 24-well plate (Falcon Cat No. 351147) and then the plates were placed at 37 °C for 30 min. Fifteen male Canton S flies of 3–5 days old were added to each well of the 24-well plate after they had been starved for 3 h. Carbon dioxide was used to an'esthetize flies for the sorting and transferring process. PBS buffer was used as negative control. Each group contained 6 wells and the experiments were repeated four times. The plates were incubated at 28 °C and 21 °C. The number of live flies was counted at 24 h interval.

Quantitative bacteriology was preformed as described previously with modification [30]. Five infected live flies from the parallel groups to each infection in Drosophila assay were crushed in 200 mL LB at 24 h interval after infection and then diluted with LB. Dilutions were spread onto PIA plates and incubated at 37 °C for 16 h. Colonies were counted following incubation and CFU/fly was calculated.

2.9. Cytotoxicity assay

Cytotoxicity of *P.aeruginosa* to eukaryotic host cells was investigated by quantitation of LDH using the non radioactive cytotoxicity test kit (Promea). Briefly, bacteria were grown in 5 mL LB

medium. The pellets were collected and resuspended to an $OD_{600} = 0.2$ with PBS. The suspensions were added to each well of a 96-well plate subconfluent HeLa cells at a multiplicity ratio of 100:1、50:1、25:1 respectively. After infection in 5% CO_2 at 37 °C for 4 h, the 50 μL supernatant was seeded into another well of a 96-well plate after centrifugation at 250 × 3g for 4 min. The 50 μL substrates were added to these wells at the same time. The plate was incubated for 30 min at room temperature. The 50 μL stop solution was added to each well. The OD of the cultures was measured at 490 nm in the Victor^3 Multilabel Plate Reader (Perkin–Elmer, Waltham, MA, USA). Experiment was carried out in quadruplicate.

2.10. LPS purification and LAL endotoxin quantitation

LPS preparations and purification were carried out according to the method of Galanos et al. [31]. Briefly, dried bacteria are suspended in 30 mL 90% phenol solution for 5 min and then added equate of 95% phenol-water solution at 68 °C for 5 min with stirring. The mixture was cooled to 4–10 °C. The aqueous phase containing lipopolysaccharide was harvested by centrifuging at 5000 r/min for 45 min. To digest nucleic acid, the extraction of crude LPS was purified for 4 h at 37 °C by adding DNase and RNase. The endotoxic activity of LPS was determined by using an LAL gel clot kit (Associates of Cape Cod). The method was performed according to the manufacturer's recommendations. The purified LPS was diluted to 10^{-7} with endotoxin-free water, the endotoxic activity was tested. Tests were carried out three times.

2.11. Swarming motility

The mobility assay was carried out as described previously [32]. The media contains 10 g/L tryptone, 5 g/L NaCl and 0.3% agar. 2 μL of overnight cultures were spotted carefully on the plates. After being incubated at 37 °C for 12 h and 21 °C for 20 h, photographs were taken with LAS-3000 Imaging System (Fujifilm, Japan).

2.12. Protein secretion measurement

Secreted type III secretion system (TTSS) effectors were analyzed by SDS-PAGE. PAO1, PAO1(Δ0011) and PAO1(Δ0011)C were grown on the inducing conditions for type III secretion system for 6 h with agitation at 37 °C. The supernatant was collected by centrifugation at 14000 *g* for 10 min. The proteins in the supernatant were precipitated by the addition of an equal volume of 100% TCA and washed with acetone consequently. The proteins were resuspended in PBS buffer, analyzed by SDS-PAGE and followed by staining with Coomassie blue [33].

3. Results

3.1. Increased susceptibility to carbapenems in PA0011 knockout mutant

In order to investigate genes involved in the resistance of *P. aeruginosa* to carbapenems, *P. aeruginosa* transposon insertion library containing more than 30000 clones was constructed and screened for mutants with altered susceptibility to three kinds of carbapenems, imipenem, meropenem and biapenem. The transposon-disrupted mutants with altered susceptibility to these antimicrobials compared with the wild type PAO1 were detected and 48 genes listed in Table 2 were identified to be potentially associated with carbapenems resistance by arbitrary PCR and consequently sequencing and sequence comparisons with published *P. aeruginosa* genome data [16]. Among them, ten individual genes and operons have been reported to be associated with antibiotic resistance, such as *mexR*, *oprD*, *mexD* etc., the rest 38 were new potential antibiotic resistance-associated genes.

Three transposon mutants inserted in PA0011 were isolated because their susceptibility to meropenem and biapenem was increased 2–3 fold compared with the wild type PAO1.

PA0011 knockout mutant PAO1(Δ0011), a mutant complement strain PAO1(Δ0011)C and PA0011 overexpression strain PAO1(pAK-0011) were constructed to investigate the role of PA0011 on carbapenems resistacne and consequent mechanism in PAO1.

The MICs of three carbapenems for the PA0011 knockout mutant PAO1(Δ0011), the complement strain PAO1(Δ0011)C, PA0011 overexpression strain PAO1(pAK-0011) and wild-type PAO1 were measured and listed in Table 3. MICs of the three carbapenems for the PAO1(Δ0011) decreased 2–3 fold and the MICs for PAO1(Δ0011)C restored to the wild-type PAO1 levels. Furthermore, the MICs for PAO1(pAK-0011) increased 2–3 fold compared with the wild-type. Similar results were obtained from disk diffusion analysis (Fig. 1). The results indicate that PA0011 plays a role in the susceptibility of PAO1 to carbapenems.

In addition, the susceptibility of PAO1(Δ0011) to gentamicin and kanamycin also changed similar to carbapenems. These results suggest that PA0011 is important to develop resistance to a wide range of antibiotics in PA.

3.2. Regulated expression of PA0011 and changed susceptibility of PAO1(Δ0011) to the kinds of antibiotics by temperature

Opportunistic pathogens have ability to adjust and respond to a wide range of environmental and host-associated conditions [34]. Microbial response to temperature changes includes variations in metabolism, outer membrane structure, chemotaxis, and other general adaptive responses. To explore whether the expression of PA0011 respond to temperature, *PA0011::luxCDABE* transcriptional fusion reporter pKD-PA0011 was constructed and introduced into the wild-type PAO1. The light produced from luxCDABE was determined both at 21 °C and 37 °C (environment and host temperature) when the bacterial reached their stationary phase. The result showed that the expression of PA0011 in PAO1 was apparently higher at 21 °C than that at 37 °C (Fig. 2A, B) Real-time qPCR was also carried out to confirm the expression of PA0011 at different temperatures (Fig. 2B). The results showed that the mRNA level of PA0011 was increased significantly at 21 °C compared with that at 37 °C ($P < 0.01$, Student's *t* Test).

Then the susceptibility of PAO1(Δ0011) was compared with the wild type PAO1 at different temperature. The MICs of PAO1(Δ0011) for impenem, meropenem, biapenem at 21 °C (0.24 ± 0.07 μg/ml, 0.12 ± 0.05 μg/ml, 0.08 ± 0.03 μg/ml) was significantly lower than those at 37 °C (0.65 ± 0.12 μg/ml, 0.25 ± 0.08 μg/ml, 0.20 ± 0.05 μg/ml) ($P < 0.05$, Student's *t* Test). Disk diffusion analysis also showed that PAO1(Δ0011) were more susceptible to the three carbapenems at 21 °C than those at 37 °C (Fig. 2C). The carbapenem susceptibility of PAO1 showed no obvious difference between at 21 °C and 37 °C.

The susceptibility of PAO1(Δ0011) to gentamicin, kanamycin and tetracycline etc. has also been compared at 21 °C with those at 37 °C by MIC determination and disk diffusion analysis. The results were similar to those for carbapenems (data not shown) except for rifampicin (data not shown). There were no obviously difference in susceptibility to rifampicin for PAO1(Δ0011) between 21 °C and 37 °C.

The above results indicated that the expression of PA0011 is temperature regulated which is higher at 21 °C than at 37 °C. PA0011 plays an important role on the resistance to the most antibiotics in a temperature dependent pattern in PAO1 except for rifampicin. There would be other gene(s) involved in rifampicin resistance which need further investigated.

Table 2
Relative susceptibility and chromosomal position of the mutations that altered the level of antibiotic susceptibility of *P. aeruginosa*.[a]

Gene	Name or function of the encoded protein	Fold-change in antibiotic susceptibility		
		Imp	Mem	Bip
PA0011***	Lauroyl/myristoyl acyltransferase(class2)	−2	−2	−2
PA0300*	*SpuD*. Spermidine/putrescine-binding periplasmic protein(class1)			+8
PA0424**	*MexR*. multidrug resistance operon repressor(class1)		+4	
PA0663*	Unknown(class4)			−2
PA0667*	Membrane proteins related to metalloendopeptidases(class4)			−4
PA0668*	23SrRNA			+4
PA0717*	Unknown(class4)		−6	
PA0844*	*PlcH*. phospholipase C activity(class1)		+2	
PA0954*	[NiFe] hydrogenase maturation protein HypF(class3)			+4
PA0958******	*OprD*. Outer membrane proteins (class1)	+8	+8	+4
PA0959*	Diadenosine tetraphosphate (Ap4A) hydrolase and other HIT family hydrolases(class4)		−3	
PA0964*	Regulation of transcription(class4)		−6	
PA0978*	Transposase and inactivated derivatives (class4)	+4	+4	+3
PA1028*	Glycine/D-amino acid oxidases(class3)			+4
PA1055*	Transport and binding proteins(class4)	−2		
PA1056***	Proton transport(class3)		−3	
PA1057*	Proton transport(class4)		−3	
PA1064*	Predicted membrane protein(class4)	−2	−2	−4
PA1150*	*pys2*. Chromosome segregation ATPases(class2)			+2
PA1180*	*phoQ*. Signal transduction histidine kinase(class1)		+1	
PA1409*	*aphA*. Deacetylases(class2)			+2
PA1538**	Predicted flavoprotein involved in K+ transport(class3)		+4	+4
PA1777****	*OprF*. Outer membrane protein and related peptidoglycan-associated proteins(class1)		−3	−2
PA2505***	*opdT*. Transport of small molecules(class1)			+8
PA2520**	*CzcA*. outer membrane protein(class2)		+2	+4
PA2522*	*czcC*. outer membrane protein precursor(class1)	+2		
PA2734*	Restriction endonuclease S subunits(class4)			+4
PA2810***	*copS*. Signal transduction histidine kinase(class1)	+4	+2	
PA2922*	Amidohydrolase(class3)			+4
PA2963*	Conserved hypothetical protein, YceG family(class4)			−2
PA3068*	*gdhB*. NAD-specific glutamate dehydrogenase(class2)	+8		
PA3165*	Amino acid metabolic process(class2)			−4
PA3223*	*AzoR3*. Acyl carrier protein phosphodiesterase(class1)		+2	
PA3257**	*Prc*. Periplasmic protease(class2)			−4
PA3265*	Membrane transporters of cations and cationic drugs(class3)	−2		
PA3620***	*mutS*. Mismatch repair ATPase(class2)		+8	+4
PA3857*	*Pcs*. Phosphatidylserine synthase(class1)		−6	
PA3901*	*fecA*. Outer membrane receptor for Fe3+-dicitrate(class2)	+6	+3	+4
PA3946*	*rocS1*. Signal transduction histidine kinase(class1)			+4
PA4393*	*AmpG*. AmpG-related permease(class1)	−3		
PA4591*	Multidrug resistance efflux pump(class4)		+1	
PA4598*****	*mexD*. multidrug efflux pump(class1)			+2
PA4639*	Unknown(class4)			+4
PA4643*	Unknown(class4)	+4		
PA4912*	Branched-chain amino acid ABC-type transport system,(class1)			+8
PA4946***	*MutL*. DNA mismatch repair enzyme(class2)		+8	+4
PA5365*	*phoU*. Phosphate uptake regulator(class1)			−2
PA5368*	*Pst*. ABC-type uncharacterized transport system, permease component(class1)		−2	

[a] *, the number of identical insertion transposon mutants; +, resistant enhancement; −, increase of susceptibility; Imp, Imipenem; Mem, Meropenem; Bip, Biapenem.

Table 3
The MICs of Imp, Mem and Bip against PAO1(Δ0011), PAO1(Δ0011)C, PAO1(pAK-0011) and PAO1 in LB broth.

Strains	MIC (μg/mL)		
	Imp	Mem	Bip
PAO (Δ0011)	0.65 ± 0.12	0.25 ± 0.08	0.20 ± 0.05
PAO1(0011)C	1.30 ± 0.20	0.60 ± 0.08	0.55 ± 0.06
PAO1(pAK0011)	2.40 ± 0.16	2.00 ± 0.05	1.50 ± 0.05
PAO1	1.32 ± 0.05	0.60 ± 0.10	0.50 ± 0.05

3.3. Impaired integrity and stability of the outer membrane in PAO1(Δ0011)

Lipopolysaccharide (LPS) creates a permeability barrier in the outer leaflet of the outer membrane (OM) of Gram-negative bacteria. The integrity and stability of the outer membrane in PAO1(Δ0011), PAO1(Δ0011)C and PAO1 were investigated as PA0011 encodes a 2-OH-lauroyltransferase response for the late acylation of lipid A.

The sensitivity of the bacterial strains to small antimicrobial compounds responsive to damaged outer membrane integrity, including polymyxin-B (MW 1302), vancomycin (MW 1449), rifampicin (MW 800) erythromycin (MW 733) were monitored [35]. The MICs of polymyxin-B, vancomycin, erythromycin and rifampicin for PAO1(Δ0011) (1.5 ± 0.05 μg/mL, 45 ± 0.03 μg/mL, 6.25 ± 0.02 μg/mL, 45 ± 0.02 μg/mL) have decreased compared with those for PAO1 (3 ± 0.03 μg/mL, 125 ± 0.05 μg/mL, 25 ± 0.03 μg/mL, 200 ± 0.02 μg/mL) ($P < 0.05$, student's t test). The MICs were restored to the wild-type level for PAO1(Δ0011)C. The results suggest that the deletion of PA0011 has impaired the integrity of the outer membrane in PAO1.

The stability of the outer membrane was subsequently determined by means of SDS-EDTA sensitivity analysis. EDTA is an ion

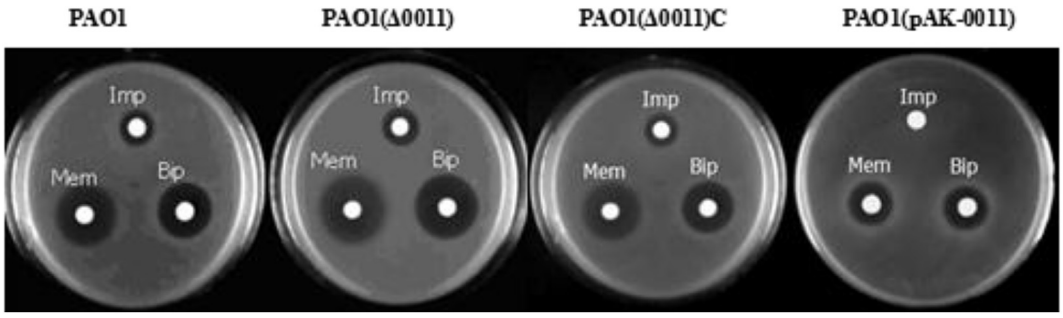

Fig. 1. Antibiotic susceptibility of PAO1(Δ0011), PAO1(Δ0011)C, PAO1(pAK-0011) and PAO1. 0.5 μg impenem (Imp), 0.5 μg meropenem (Mem) and 0.5 μg biapenem (Bip) were added to the filter disks.

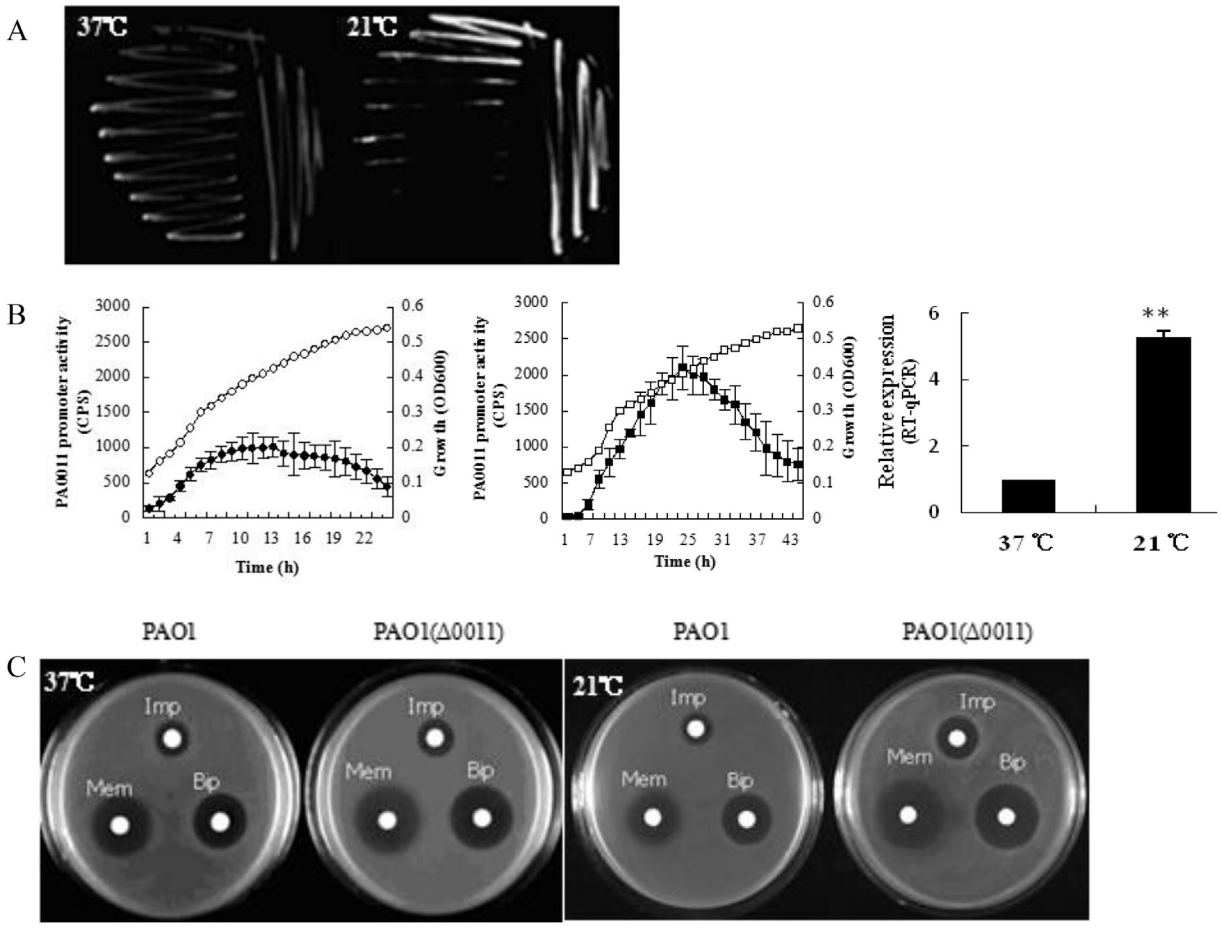

Fig. 2. Temperature influence the expression of PA0011 and the susceptibility of PAO1(Δ0011) to the carbapenems. A. The expression analysis of PA0011 at 37 °C and 21 °C by using *PA0011::luxCDABE* transcriptional fusion reporter in PAO1. B. The promoter activity of PA0011 in PAO1 at 37 °C(triangles) and 21 °C(squares). Expression profiles and corresponding growth curves are shown by solid and open symbols respectively. The results represent the average of triplicate experiments and the error bars indicate standard deviations; The mRNA level of gene PA0011 was normalized to that of *proC* in the wild strain PAO1. Results are shown as relative expression ratios at 21 °C compared to expression at 37 °C. C. Antibiotic susceptibility for PAO1(Δ0011) and the wild type PAO1 at 37 °C and 21 °C. 0.5 μg impenem (Imp), 0.5 μg meropenem (Mem) and 0.5 μg biapenem (Bip) were added to the filter disks.

chelator which chelates divalent cations and interrupts intermolecular associations and therefore disturbs the stability of the outer membrane. PAO1(Δ0011), PAO1(Δ0011)C and PAO1 were grown on LB agar medium containing 0.5% SDS with varying amounts of EDTA. At the concentrations lower than 1.6 mM, no growth difference was observed among these strains (data not shown). However, at a concentration of 1.6 mM, even though EDTA had no effect on the viability of either PAO1 or PAO1(Δ0011)C, the viability of PAO1(Δ0011) was completely absent (Fig. 3A). The stability of the outer membrane was also determined at 21 °C. The viability of PAO1(Δ0011) was completely absent at 1.4 mM EDTA at 21 °C (Fig. 3B), which is lower than that at 37 °C. However, the inactivation of PAOO11 had no effect on the growth of PAO1(Δ0011) compared with wild type at the two temperatures (Fig. 3A, B).

The results demonstrated that the inactivity of PA0011 compromised the integrity and decreased the stability of the outer membrane in PAO1.

3.4. Decreased cytotoxicity in PA0011 mutant

The structural integrity of OM was required for bacterial pathogenesis to mammalian cell [36]. The inactivity of PA0011 impaired the integrity and stability of the OM in PAO1. Then cytotoxicity was assayed by the CytoTox 96 Non-Radioactive Cytotoxicity test kit (Promea). As presented in Fig. 4, the LDH release of HeLa cells infected by PAO1(Δ0011) was similar to the HeLa cells spontaneous release without bacterial infection ($P > 0.1$, Student's t test). The PAO1 and PAO1(Δ0011)C exhibited significantly more cytotoxicity than PAO1(Δ0011) ($P < 0.01$, T test). There was no difference in cytotoxicity between PAO1 and PAO1(Δ0011)C ($P > 0.1$, Student's t test) . The results indicated that the deletion of PA0011 caused less cytotoxicity to HeLa cells compared with wild-type PAO1.

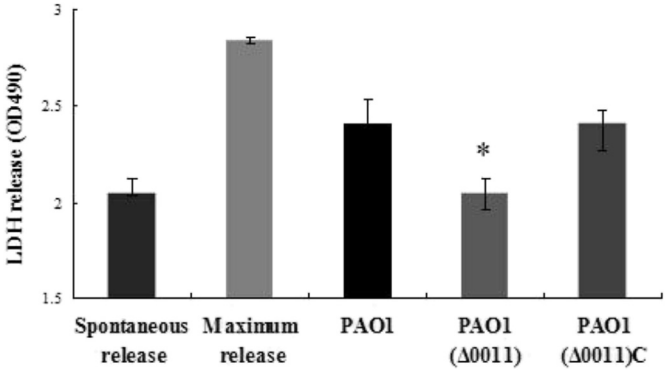

Fig. 4. Cytotoxicity assay. LDH release by HeLa cells infected with PAO1, PAO1(Δ0011) and PAO1(Δ0011)C. Series 1 represents HeLa cells spontaneous release and Series 2 represents HeLa cells maximum release. Result represents the average of triplicate experiments. Error bars indicate one standard deviation.

3.5. Attenuated virulence by the inactivity of PA0011

To investigate the role of PA0011 on pathogenicity in PAO1, the virulence analysis was monitored via a *Drosophila* feeding assay. The virulence of PAO1(Δ0011) was compared with that of the wild type PAO1 and with the complementary strain PAO1(Δ0011)C. *Drosophila melanogaster* Canton S infected with PAO1(Δ0011) showed higher survival rate compared with those infected with PAO1 ($P < 0.01$, Log-rank test) at room temperature(28 °C) (Fig. 5b). There was no significant difference between PAO1 and PAO1(Δ0011)C ($P > 0.1$, Log-rank test).

Although the inactivation of PA0011 does not impact the growth of PAO1 *in vitro* (Figs. 3 and 8A), the growth of the strains *in vivo* was still determined by CFU analysis in our drosophila assays to further investigate the changes in virulence. There is no significant difference on the growth among the mutant, the complement and wild type strains (Fig. 5C). The results suggested that the reduced virulence is not caused by the growth difference *in vivo*.

A Chinese cabbage (*B. pekinensis*) infection model was used to further investigate the influence of PA0011 on pathogenicity. PAO1(Δ0011) caused obviously less severe necrosis and maceration than PAO1 at 37 °C (Fig. 5A). There were no obvious difference between the wild type PAO1 and the complementary strain PAO(Δ0011)C (date not show).

The virulence of PAO1 and PAO1(Δ0011) at 21 °C were also monitored and the results are similar to those at 37 °C shown in Fig. 5A and B. Moreover, the virulence of PAO1(Δ0011) at 21 °C was much less than that of PAO1 at 37 °C and 28 °C both with *B. pekinensis* infection model and *Drosophila* feeding assay respectively.

The results above suggested that PA0011 is involved in the virulence of PAO1 with temperature-dependent pattern. The inactivation of PA0011 reduced the virulence of PAO1.

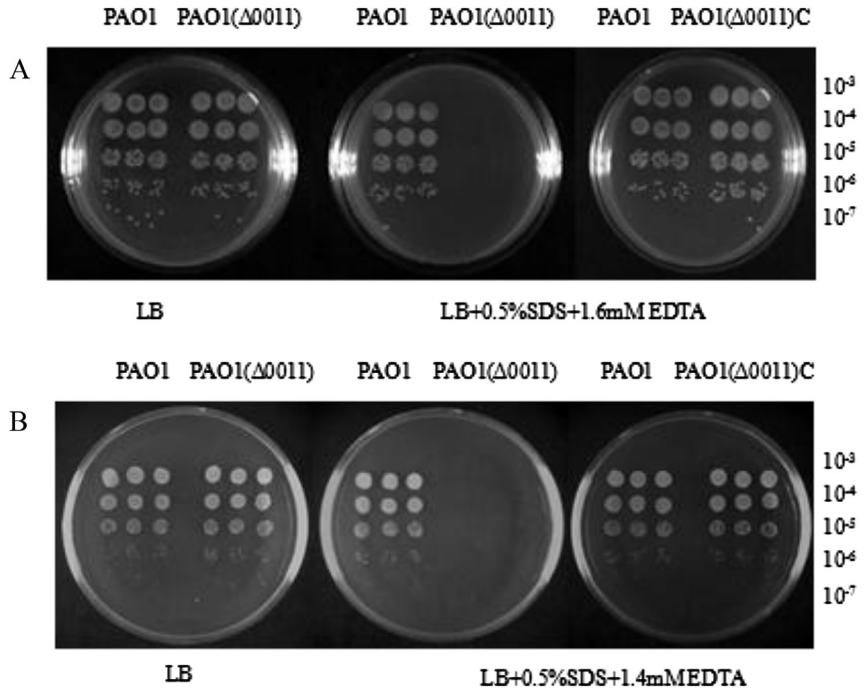

Fig. 3. SDS-EDTA sensitivity profiles of PAO1, PAO1(Δ0011) and PAO1(Δ0011)C in LB agar plates A. with or without 0.5% SDS and 1.6 mM EDTA at 37 °C, B. with or without 0.5% SDS and 1.4 mM EDTA at 21 °C.

3.6. Decreased endotoxic activity in PA0011 knockout mutant

Lipid A is the endotoxin component of LPS. The disruption of PA0011 reduced the pathogenicity of *P. aeruginosa*. To verify whether the attenuated virulence of the PAO1(Δ0011) could be related to the reduction of endotoxic activity, LPS were extracted and purified from PAO1, PAO1(Δ0011) and PAO1(Δ0011)C cultivated both at 21 °C and 37 °C respectively. The endotoxic activity of LPS both from 37 °C to 21 °C were determined for PAO1, PAO1(Δ0011) and PAO1(Δ0011)C. As presented in Fig. 6, the endotoxic activity of LPS from PAO1(Δ0011) at 37 °C was 0.89×10^8 EU/mL, while the activity of LPS from PAO1 was 1.90×10^8 EU/mL ($P < 0.05$, T test). PAO1(Δ0011)C restored the endotoxic activity to the level of PAO1(1.92×10^8 EU/mL) ($P > 0.01$, Student's t test). The situation at 21 °C was similar and the endotoxic activity of LPS from PAO1(Δ0011) at 21 °C was much more lower than that of PAO1(Δ0011) at 37 °C.

The results indicated that deletion of PA0011 decreased the endotoxic activity of LPS in PAO1. This effect could probably cause the attenuated virulence in the mutant at least partially.

3.7. Decreased swarming motility in PA0011 knockout mutant

Motility is supposedly a very ancient property of bacteria and acts as an important virulence factor [37]. Alterations in LPS could interfere the motility of flagella-associated in *Yesinia*, *E. coli and Myxococcus xanthus* [38–40]. The late Lipid A acyltransferases play an impotant role on the Lipid A acylation in the biosynthesis of LPS.

P.seudomonas has possessed two kinds of surface organelles including a single polar flagellum responsible for swimming motility in liquid environments and polar type IV pili (TFP) contribute to twitching motility across solid surfaces. Swarming motility is usually related to changes in flagellar number as well as placement in semi-solid surfaces.

The motility of PAO1, PAO1(Δ0011) and PAO1(Δ0011)C was monitored. The swarming motility of PAO1(Δ0011) was evidently decreased compared with that of PAO1 or PAO1(Δ0011)C at both 37 °C and 21 °C(Fig. 7). However, there were no difference on swimming and twitching between the mutant and the wild type (data not shown).

The inactivation of PA0011 may have a great influence on the flagellar number and placement rather than other structures associated with swimming and twitching motility. Whether the decreased swarming motility contribute to the reduced cytotoxicity and virulence of PAO1(Δ0011) need further investigation.

3.8. Impacts of the expression and secretion of TTSS proteins by PA0011

The type III secretion system (TTSS) is an export system used by several pathogens to secrete effector proteins into the extracellular milieu or inject them into the membranes or cytosol of eukaryotic

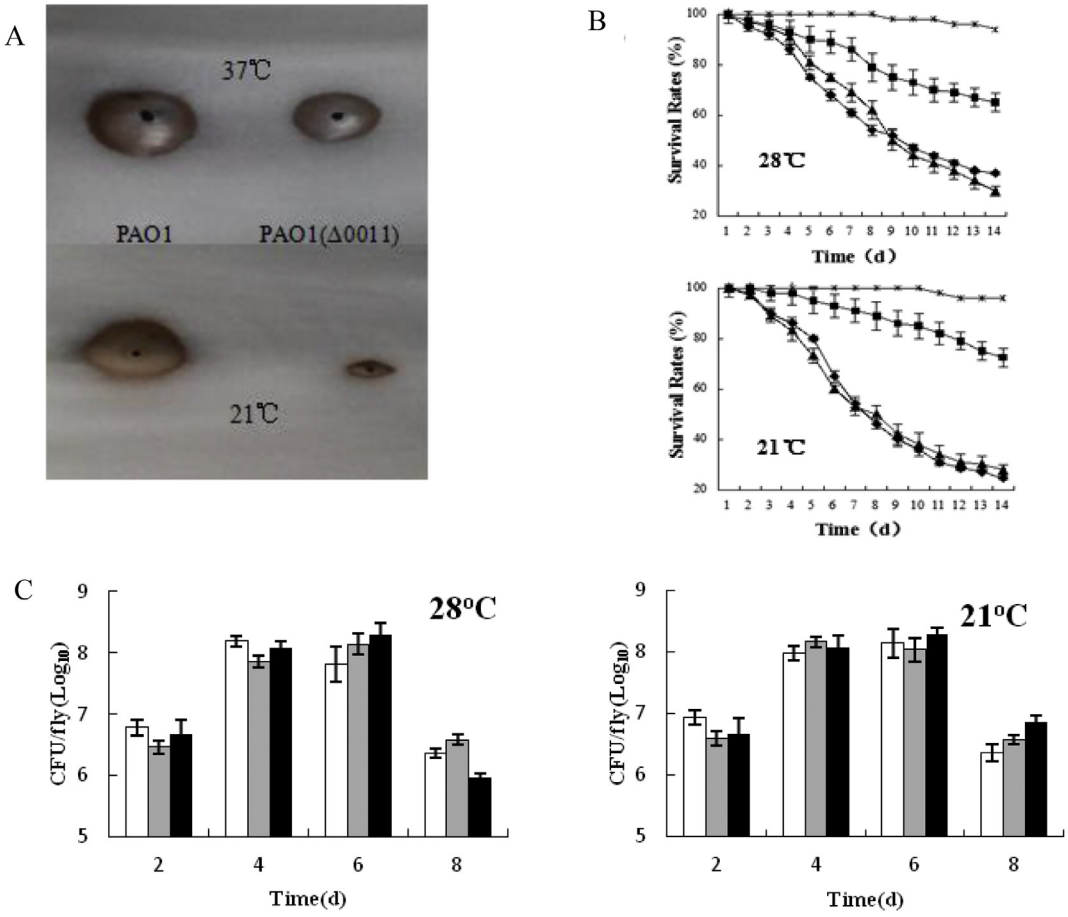

Fig. 5. Virulence analysis of PAO1 (Δ0011) and PAO1 in *Brassica pekinensis* and *Drosophila melanogaster* Canton S hosts. A. Necrosis and maceration caused by PAO(Δ0011) and PAO1 in Brassica pekinensis at 37 °C and 21 °C. B. Survival rates of Drosophila melanogaster Canton S infected with PAO1 (diamonds), PAO(Δ0011) (squares) and PAO(Δ0011)C (triangles) at 28 °C and 21 °C, PBS was used as an uninfected control (asterisks). C. CFU analysis for the infection group with PAO1 (white), PAO1(Δ0011) (grey) and PAO1(Δ0011)C (black) respectively. The experiments were repeated five times. The data shown are average numbers.

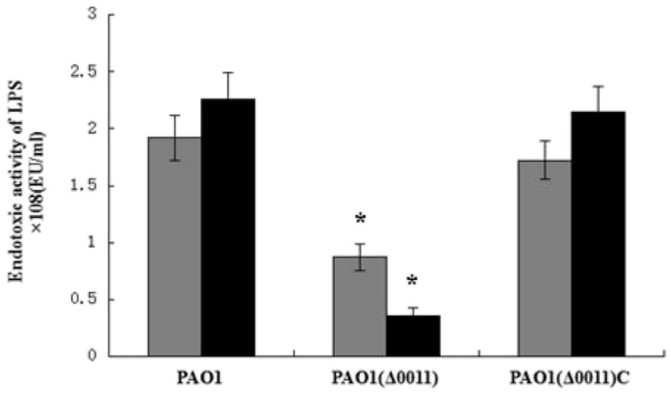

Fig. 6. Endotoxic activity of LPSs isolated from wild-type PAO1, PAO1(Δ0011) and PAO1(Δ0011)C at both 37 °C (grey) and 21 °C (black). Result represents the average of triplicate experiments. Error bars indicate one standard deviation.

cells [41]. The LPS status affects the expression of the type III secretion systems which mediates bacterial invasion into host cells [42–44]. PA0011 coding a late acyltransferases participates in the LPS biosynthesis and its inactivation impaired integrity of outer membrane and decreased endotoxic activity of LPS in *P. aeruginosa*. In order to assess the influence of PA0011 on the TTSS system, the expression of *exsC*, *exsD*, *exoS*, *exoY* and *exoT*, effectors of TTSS, was determined both in PAO1 and PAO1(Δ0011) by using the lux-based reporter.

The expression profiles of *exsD* and *exoY* in PAO1(Δ0011) and PAO1 are shown in Fig. 8A. No growth difference was observed between the mutant and the wild type. Interestingly, the expression of *exsD* and *exoY* in PAO1(Δ0011) are much higher than those in wild-type PAO1 ($P < 0.05$, Student's *t* test). The situations of other TTSS effectors, *exoS* and *exoT*, are similar to those shown in Fig. 8A (data not shown). Real-time qPCR was carried out to confirm the expression of *exsD*, *exoS* and *exoT* in PAO1 and PAO1(Δ0011). The results indicated that the mRNA levels of *exsD*, *exoS* and *exoT*, were increased significantly in PAO1(Δ0011) compared with PAO1 ($P < 0.05$, Student's *t* test) (Table 4).

The effect of PA0011 on TTSS was also confirmed by examining the secretion of TTSS effectors under TTSS inducing conditions as shown in Fig. 8B. The secreted proteins of approximate 53 kDa and 49 kDa, corresponding to ExoT and ExoS respectively, were higher in PAO1(Δ0011) than those in PAO1 and PAO1(Δ0011)C. PAO1(Δ0011)C restored the secretion to the level of PAO1.

These results suggested the existence of a cooperative association between Lipid A and the TTSS in *P. aeruginosa*.

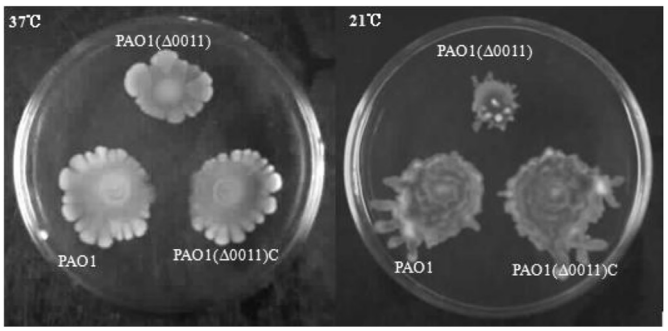

Fig. 7. Swarming motility of PAO1, PAO1 (Δ0011) and PAO1 (Δ0011)C on swimming semisolid agar plates at both 37 °C and 21 °C. The experiment was repeated three times.

4. Discussion

PA0011 has been found to affect the expression of *phzA1B1C1-D1E1F1G1* (*phzA1*) operon in the presence of sub-inhibitory concentration (SIC) of tetracycline in our previous report [14]. Interestingly, the PA0011 transposon insertion mutant was found probably involved in carbapenem resistance in PAO1 during our screening of a transposon insertion library of *P. aeruginosa* with three kinds of carbapenems, impenem, meropenem and biapenem. Therefore the role of PA0011 on the resistance to the antibiotics and other characters have been investigated further in this study.

The first aim of the study was to determine the role of PA0011 on the carbapenem resistance in PAO1. By constructing PA0011 mutant PAO1(Δ0011), the complementary strain PAO1(Δ0011)C and over-expression strain PAO1(pAK-0011), the susceptibility analysis by MICs and disk diffusion measurement showed that PA0011 is involved in the carbapenems resistance in PAO1(Table 3, Fig. 1). The involvement of PA0011 on the resistance to gentamycin and kanamycin has also been proved in this study (data not shown).

PA0011 is a 2-OH-lauroyltransferase coding gene homologous to *lpxL* (*htrB*) and *lpxM* (*msbB*) from *E. coli*, but exhibits higher identity to *lpxL* which encodes a secondary acyltransferase HtrB to catalyze addition of the secondary laureate (C_{12}) to Kdo_2-lipid A (lipid IV_A) in lipid A biosynthesis in *E. coli*. *lpxM* encodes a secondary acyltransferase MsbB to catalyze addition of the secondary myristate (C_{14}) to Kdo2-lipid A in *E. coli*. Another kind of acyltransferase, encoding by *lpxP* is responsible for the addition of palmitoleate (C_{16}) at 12 °C in *E. coli*. Lipid A, acting as the hydrophobic anchor of LPS in the outer surface of the outer membrane [11–13], consists of a D-glucosamine disaccharide phosphated at positions 1 and 4 and acylated at several different positions by first as well as second acylation in different species [10]. Although the biochemical synthesis of lipid A is generally conserved, the different modification of lipid A by detaching the phosphate groups or modifying acyl chains could mediate wide variations in different species [45]. In many pathogens, such as *Klebsiella pneumoniae*, *E. coliK12* and *Salmonella typhimurium*, secondary acylation of lipid A is linked to resistance to antimicrobial peptides [46–48]. Our results indicated that PA0011 plays an important role on the resistance to not only the antimicrobial peptides but also carbapenems, gentamycin and kanamycin.

Furthermore, we have found that the involvement of PA0011 in the antibiotics resistance is temperature-regulated. The transcriptional expression of PA0011 at 21 °C was higher than that at 37 °C (Fig. 2A, B) and the verified expression of PA0011 caused consequently changed susceptibility of PAO1 and PAO1(Δ0011) to the antibiotics except for rifampicin (Fig. 2C). There could be some other gene(s) responsible for the resistance of rifampicin at 21 °C because PA0011 is involved in the resistance to rifampicin but the susceptibility of both PAO1 and PAO1(Δ0011) showed no difference between at 21 °C and 37 °C. The expression of PA0011 regulated by temperature is similar to that of *msbB* (*lpxP*) which encodes an acyltransferase responsible for the addition of C_{12} in *Yersinia enterocolitica* [38]. But the regulated expression of *msb*B dose not caused the consequent susceptibility variation because the *msbB* mutant shows more susceptibility to polymyxin-B at 37 °C than that at 21 °C and there is no difference on the susceptibility between the *msb*B mutant and the wild type at 21 °C [38]. *htrB* (*lpxM*), coding acyltransferase responsible for the addition of C_{14} in *Y. enterocolitica*, is also temperature-regulated, higher expression at 37 °C than at 21 °C. *htrB* mutant shows more susceptibility to polymyxin-B than that of the wild type at 37 °C but the same resistance as the wild type at 21 °C. *htrB*(*lpxM*) in *E. coli* is required for the addition of C_{12} at 37 °C [49]. In *E. coli* or *S. typhimurium* cells cultured below 15 °C, palmitoleate acyltransferase LpxP replaces

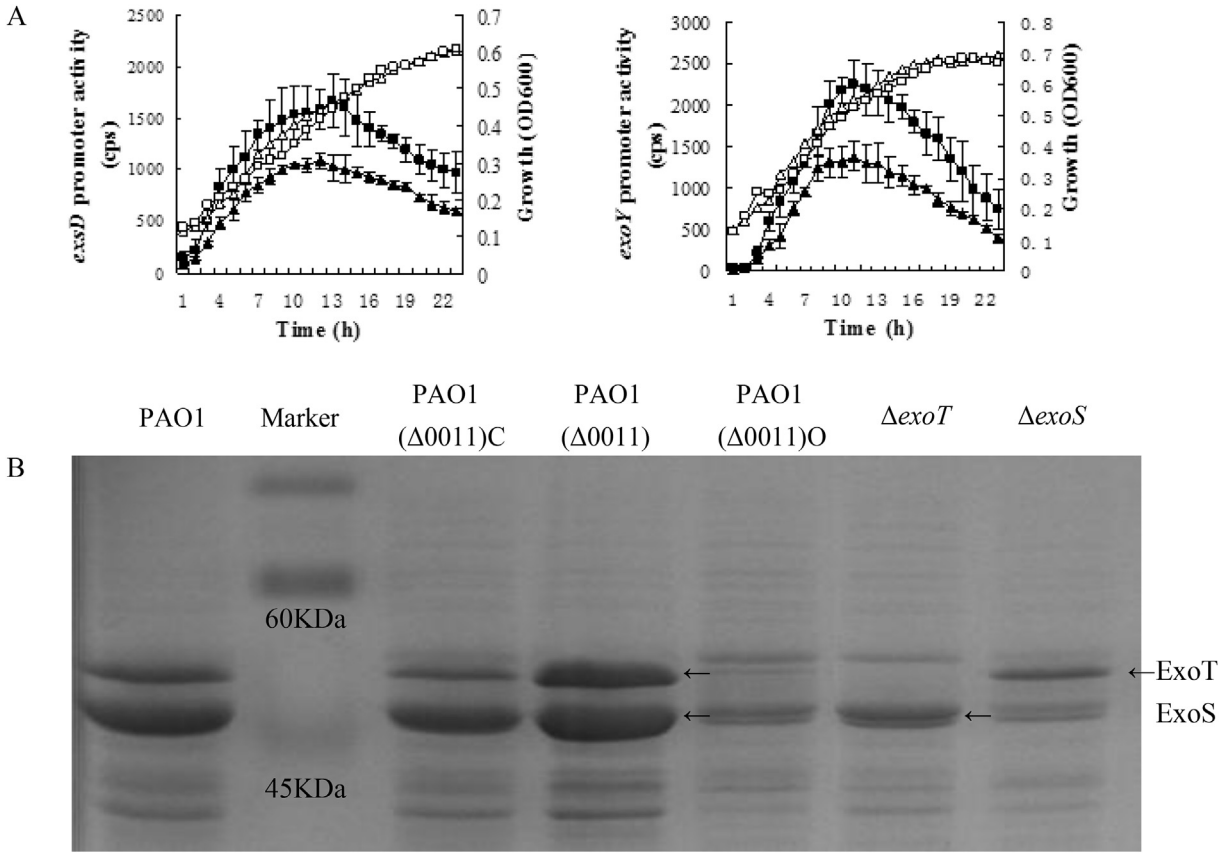

Fig. 8. Effect of PA0011 on the expression of T3SS effectors secretion. A. The promoter activity of *exsD* and *exoY* in PAO1(Δ0011) (squares) and PAO1(triangles). Expression profiles and corresponding growth curves are shown by solid and open symbols respectively. The results represent the average of triplicate experiments and the error bars indicate standard deviations. B. Effect of PA0011 on T3SS effectors secretion. Bands corresponding to T3SS effectors (ExoT and ExoS) are indicated by arrows.

Table 4
The transcriptional levels of selected genes in PAO1 and PAO1(Δ0011).

Genes	The level of mRNA[a]		
	PAO1	PAO1(Δ0011) (Mean ± SD)[b]	*p* value
exoS	1.00	3.26 ± 0.04	0.031
exoT	1.00	6.34 ± 0.05	0.009
exsD	1.00	2.97 ± 0.04	0.024

[a] The mRNA level of each gene was normalized to that of *proC*. Results are shown as relative expression ratios compared to expression in the wild strain PAO1.
[b] Each value shown represents the mean ±(SD) of three different determinations.

the laurate acyltransferase coding gene *htrB* that is normally linked to lipid A in cells grown at 30–42 °C, however, expression of *lpxM*, coding the myristoyl transferase, is not changed at lower temperature [50]. These reports indicate that acyltransferases in different species were differently affected by temperature. PA0011 is different from any acyltransferase coding genes in a temperature regulated pattern. The expression of *PA0011* with a temperature regulated pattern may be involved in bacterial adaptation to environmental conditions.

The highly ordered structure of the LPS layer not only provides protection to the cell as a physical barrier but also contributes an effective permeability barrier against external noxious agents to the outer membrane of gram-negative bacteria. The inactivation of PA0011 impaired the integrity and stability of the outer membrane in *P. aeruginosa* (Fig. 3). That caused the consequently increased susceptivity to antibiotics at least partially. The out membrane of PAO1(Δ0011) showed less stable at 21 °C (Fig. 3B) than that at 37 °C (Fig. 3A). Thus PAO1(Δ0011) was more susceptible to the three carbapenems at 21 °C than those at 37 °C. The inactivation of *lpxM* in *K. pneumoniae* and *lpxL* in *E. coli* increased permeability of the outer membrane [48,51]. However, the permeability barrier of the OM was not altered in acyltransferase mutants in *Y. enterocolitica* [38]. These could probably be caused from the different species and the specific lipid A situation. In *P. aeruginosa*, lipid A is formed by a, β(1′,6)-linked disaccharide of glucosamine acylated with R-3-hydroxydodecanoic groups at the 2, 3, 2′ and 3′ positions. Its 3-hydroxyl group of the two amide-linked 12:0(3-OH) residues are further acylated by either dodecanoic (12:0) or (S)-2-hydroxydodecanoic acid catalyzed by the late 2-OH-lauroyltransferase [10] which is different from those in *E. coli* and other species. Moreover, the late acyltransferases of *P. aeruginosa* and *Neisseria meningitidis* do not require the Kdo disaccharide for enzymatic activity and transfer one laurate (C12:0) chain to each glucosamine unit at the 2- and 2′-positions to form a more symmetrical lipid A [52,53].

P. aeruginosa shows low-level susceptibility to a wide variety of antibiotics due to its intrinsic resistance. This intrinsic mechanisms has been attributed to the low permeability of cellular envelops and the specific detoxification such as multidrug efflux pumps or antibiotic inactivating enzymes. All these elements presenting in the core-genome of bacteria is hard-wired into the cell other than just avoiding the activity of a given antibiotic [54]. The involvement of PA0011 in the permeability and stability of cellular outermembrane (OM) and the consequent resistance to most of antibiotics used in clinic nowadays suggest that PA0011 plays a key role on the emergent property of the system in *P. aeruginosa*.

The lipid A has been studied as a possible virulence determinant in a number of Gram-negative bacteria [46,47,55]. Late(secondary) acylation of lipid A is linked to not only resistance to antimicrobial peptides but also the virulence in many pathogens [45]. In *E. coli K12*, *S. typhimurium* and *K. pneumoniae*, *lpxM* mutants showed reduced virulence in animal models [46–48]. However, *lpxM* (msbB) mutant was not related to virulence in *Y. enterocolitica* [38].

The second aim of the study was to investigate the role of PA0011 on virulence in PAO1.

In this report, PA0011 has been found involved in the virulence in PAO1 because PA0011 mutant showed obviously decreased cytotoxicity (Fig. 4) and infection ability both *in vivo* and *in vitro* infection models (Fig. 5A, B). Furthermore, the virulence of PAO1(Δ0011) at 21 °C was much less than that of PAO1 compared with the situation at 37 °C both in *B. pekinensis* infection model and *Drosophila* feeding assay(Fig. 5A, B). Together with the antibiotics resistance results, we found that the inactivation of PA0011 caused a greater impact on both resistance and virulence at 21 °C than those at 37 °C. The expression of PA0011 at 21 °C was higher than that at 37 °C, which suggested that the up-regulated PA0011 is important for *P. aeruginosa* to survive or grow at low temperature. It has been validated that deacylation can increase acyl chain fluidity [56]. Therefore the hypoacylation of Lipid A caused by the decreased PA0011 expression at body temperature could be benefit to the acyl chain fluidity. The increased acyl-chain fluidity is known to play an important role on facilitating the exchange of hydrophobic nutrients and metabolites within host and invading host tissues [57]. Thereby the temperature-dependent regulation of PA0011 acts an important role for the cell to response to different environment temperature in PAO1.

The inactivation of PA0011 does not influence the growth of the mutant both *in vitro* (Figs. 3 and 8A) and *in vivo* (Fig. 5C). Therefore, the reduced infection ability was not caused by the different growth between the wild type and the mutant. The reduced endotoxic activity of LPS could contribute to the decreased virulence both *in vivo* and *in vitro*. The LPS from PAO1(Δ0011) was 2-fold less toxic than that from the wild type PAO1 *in vitro* (Fig. 6), similar to the situation of *lpxM* mutants in *Salmonella* and *Haemophilus influenzae* [35,58]. The reduced toxicity may partially account for decreased virulence of the mutant.

In the same time, inactivity of PA0011 also reduced the swarming motility significantly compared with wild type PAO1 (Fig. 7), similar to the situation of *msbB* and *lpxP* mutants in *Y. enterocolitica* [38]. But the inactivation of PA0011 did not influence the motility of swimming and twitching in PAO1. The result suggested PA0011 may have a great influence on the flagellar number and placement rather than other structures associated with swimming and twitching motility which need further investigation. Whether the decreased swarming motility contributes to the reduced cytotoxicity and virulence in PAO1(Δ0011) need also be searched in details.

One of the important role of LPS is to protect the bacterium from serum-mediated killing and the phagocytosis of the phagolysosome [59]. Lipid A, the specific and sensitive recognition by the innate immune system, is thought to be the primary inflammatory component of LPS [48]. The assay of phagocyte-mediated killing and the human serum sensitivity shown that the viable numbers of PAO1(Δ0011) was almost equivalent with PAO1 (date not show). These results suggested that PA0011 could probably not play a key role for *P. aeruginosa* to resist the innate immune system, but further studies to test the influence of the acylation pattern of lipid A on innate immune recognition and stimulation need ongoing.

TTSS is one of the principal surface-associated virulence factors of *P. aeruginosa*. D. K. Augustin et al. have found that the loss of LPS O antigen (particularly the highly structured B band) would facilitate secretion of the TTSS in both laboratory and clinical isolates of *P. aeruginosa* [44]. Our result indicated that inactivation of PA0011 increased the expression and secretion of TTSS effectors in *P. aeruginosa* (Fig. 8, Table 4). However, the secretion of TTSS effectors is impaired in *msbB* (*lpxM*) mutant in *Salmonella* [60], while the secretion of TTSS effectors is not altered in the *msbB*, *lpxP* or *htrB* mutants in *Y. enterocolitica* [38]. The different effect of lipid A acyltransferases on TTSS could probably due to the different species and the specific lipid A situation.

G. Singh et al. have reported that the secretion of TTSS effectors in *P. aeruginosa* is negatively regulated by the PQS concentration [61]. Our previous study showed that PA0011 regulates the *rhl*/*las* systems negatively [62]. *rhl* and *las* systems exert negative and positive regulation on PQS respectively [63]. D. K. Augustin et al. supposed that LPS structural composition may act as a signal to regulate TTSS gene expression [44]. Based on these observations, it is possible that the inactivation of PA0011 mediates a variation of LPS, varied LPS acts as a signal to regulate *rhl* system negatively and decrease the expression of PQS consequently. Finally, low concentration of PQS promotes TTSS effectors secretion. However, the *las* and *rhl* systems as well as the PQS system have previously been associated with the inhibition of type III secretion system [61], while there are complex and reciprocal regulations between *las*/*rhl* system and PQS. We have proposed that the PQS system regulates the las/rhl system positively via its negative regulation on PA0011 in our previous report [46]. Therefore, the presence of these sophisticated communication system doesn't rule out other possibilities involved in the regulation of PA0011 on TTSS, which needs further study.

5. Conclusions

Conclusionly, PA0011 has been found involved in the virulence and resistance to kinds of antibiotics used nowadays, including carbapenem, in temperature regulated pattern. The impaired integrity and decreased stability of outer membrane in PA0011 mutant contribute to the reduced antibiotics resistance at least partially. PA0011 is also involved in the virulence with a temperature dependent pattern. The participation of PA0011 in virulence could be partly dependent on the reduced endotoxic activity of LPS. PA0011 could not play a key role on the resist to the innate immune system in *P. aeruginosa*. Additionally, PA0011 represses the expression of TTSS virulence factors both at transcriptional and translational levels. That the typical influence of PA0011 on both resistances to most antibiotics used nowadays and virulence in *P. aeruginosa* makes it a target for novel antimicrobial therapies.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Glossary

LPS	lipopolysaccharide
PAO1	*P. aeruginosa*
CF	cystic fibrosis
HIV	human immunodeficiency virus

TTSS	type III secretion system
PIA	Pseudomonas isolate agar
LB	Luria–Bertani
Kan	kanamycin
Gm	gentamicin
Amp	ampicillin
Tc	tetracycline
Tmp	trimethoprim
SDS	Sodium Dodecyl Sulfonate
EDTA	Ethylene Diamine Tetraacetic Acid
LAL	Limulus amebocyte lysate
LDH	lactate dehydrogenase
OM	outermembrane

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