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CmpX Affects Virulence in *Pseudomonas aeruginosa* Through the Gac/Rsm Signaling Pathway and by Modulating c-di-GMP Levels

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Abstract *Pseudomonas aeruginosa* is an ubiquitous organism which is able to infect and colonize many types of hosts including humans. Colonization of *P. aeruginosa* in chronic infections leads to the formation of biofilms, which are difficult to eradicate. *P. aeruginosa* is capable of regulating its virulence factors in response to external environment triggers and its signaling mechanism involves two-component regulatory systems and small molecules such as bis-(3′–5′)-cyclic dimeric guanosine monophosphate. PA1611-RetS-GacS/A-RsmA/Y/Z is a key regulatory pathway in *P. aeruginosa* that controls several virulence factors and biofilm formation. We have previously identified a conserved cytoplasmic membrane protein *cmpX* (PA1775), as a regulator for PA1611 expression. In this study, we demonstrate that *cmpX* regulates virulence, and controls biofilm formation in *P. aeruginosa* as well as provide evidence showing that *cmpX* affects Gac/Rsm pathway, possibly by modulating intra-cellular c-di-GMP levels. A *cmpX* knockout showed significantly decreased promoter activity of *exoS* (PA1362) and increased activity of small RNA, RsmY. As compared to the wild-type PAO1, *cmpX* mutant had elevated intracellular c-di-GMP level as measured indirectly by *cdrA* (PA4625) activity, as well as increased expression of *wspR* (PA3702), a c-di-GMP synthase. The transcription of the major outer membrane porin gene *oprF* (PA1777), and sigma factor *sigX* (PA1776) was also significantly decreased in the *cmpX* mutant. Biolog phenotype microarray experiments further indicated that the *cmpX* knockout mutant had increased sensitivity to membrane detergents and antibiotics such as lauryl sulfobetaine, tobramycin, and vancomycin. These results point to a significant role of *cmpX* in *P. aeruginosa* virulence and colonization.

Keywords *Pseudomonas aeruginosa* · *cmpX* · Phenotype microarrays · c-di-GMP · Biofilm

Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic human pathogen. Infection by *P. aeruginosa* is characterized by two distinct phases: acute and chronic. During acute phase of infection, *P. aeruginosa* cells are mostly planktonic with high motility and are characterized by active secretion of toxic effectors such as ExoS, ExoY, ExoT by the type three secretion system (T3SS); whereas chronic infection involves exopolysaccharide production, type six secretion system (T6SS) to overcome inter-bacterial competition (Wilton et al. 2016) and the formation of biofilms.

Under the conditions that favor either acute or chronic infection, a range of virulence genes are regulated by two-component regulatory systems (TCSs), the second messenger bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Lee et al. 2007; Moscoso et al. 2011) and cell density-dependent quorum-sensing (QS) systems such as the LasR/I and RhlR/I. PA1611-RetS-GacS/GacA-RsmA/Y/Z is a key TC regulatory pathway involved in virulence regulation during the switch between acute and chronic infection phases. PA1611 and RetS are hybrid histidine kinase (HHK) (Chambonnier et al. 2016; Kong et al. 2013) and GacS/GacA

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is a classical TCS. Under chronic infection conditions, PA1611 binds to RetS (Bhagirath et al. 2017); GacS phosphorylates GacA and activates production of small RNAs, *rsmY* and *rsmZ* (Goodman et al. 2004). RsmY and RsmZ bind and sequester transcriptional regulator RsmA (PA0905) in a titration-dependent manner to render it unavailable to activate downstream target genes. RsmA has been shown to post-translationally regulate T3SS, Type IV pili, motility, and biofilm formation in a dose-dependent manner (Brencic and Lory 2009).

Signaling through small molecules such as c-di-GMP is another important mechanism for virulence regulation in *P. aeruginosa* (Romling et al. 2013). C-di-GMP is a second messenger used by *P. aeruginosa* and many other bacteria to regulate the expression of genes associated with flagellar motility, Type IV pili and biofilm initiation. Previous studies have shown that, when free swimming *P. aeruginosa* cells attach to a solid surface, intracellular levels of c-di-GMP increase (Ha and O'Toole 2015); however, the exact cues that are sensed are yet unknown.

The two distinct mechanisms of TCS regulation and signaling via c-di-GMP intersect at several levels. Earlier, it has been shown that a diguanylate cyclase (c-di-GMP synthase) encoding *wspR* is involved in switch between T3SS and T6SS via RetS. A *retS* knockout in PAO1 demonstrated elevated c-di-GMP levels (Moscoso et al. 2011). It was also shown that small RNAs, RsmY, and RsmZ are necessary for this c-di-GMP-related response (Moscoso et al. 2011).

Previously, we have identified that a transposon inserted in *cmpX* activated the expression of PA1611 (Kong et al. 2013). In this study, we constructed a *cmpX* deletion (ΔPA1775) mutant and characterized its role in virulence regulation in *P. aeruginosa* PAO1. To confirm the role of *cmpX* in GacS/GacA-RsmA/Y/Z pathway, we studied the effects of the *cmpX* mutant on the promoter activities of T3SS effector *exoS* and small RNA *rsmY*, as well on motility and biofilm formation. Further, to see if c-di-GMP was involved in *cmpX* mediated regulation, we evaluated the effect of a *cmpX* knockout on *cdrA* and *wspR* expression levels as an indirect measure of intracellular c-di-GMP levels. Finally, we examine the effect of *cmpX* knockout on PAO1 by phenotype microarrays. We also demonstrate that two key regulatory genes *sigX* and *oprF* in *Pseudomonas* are downregulated in a *cmpX* knockout. Results indicating that *cmpX* affects both PA1611-RetS-GacS/GacA-RsmA/Y/Z and *sigX*-OprF pathways are presented.

Materials and Methods

Bacterial Strains and Plasmids

Bacterial strains and plasmids in this study are described in Table 1. *P. aeruginosa* and *Escherichia coli* were routinely grown on Luria–Bertani (LB) agar or in LB broth at 37 °C unless otherwise specified. LB was used as a T3SS non-inducing medium and LB supplemented with 5 mM EGTA and 20 mM $MgCl_2$ as a T3SS inducing medium (calcium-depleted). Antibiotics were used at the following concentrations: for *P. aeruginosa*, tetracycline (Tc) at 70 μg/ml in LB or 300 μg/ml in Pseudomonas Isolation Agar (PIA), carbenicillin (Cb) at 250 μg/ml and trimethoprim (Tmp) at 300 μg/ml in LB; for *E. coli*, kanamycin (Kn) at 50 μg/ml and ampicillin (Ap) at 100 μg/ml in LB.

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 (Duan et al. 2003). For measuring the promoter activity of *sigX* and *oprF,* plasmid pMS402 carrying a promoterless *gfp* gene was used to construct promoter-*gfp* fusions. Promoter regions of *exoS*, *rsmY, sigX, oprF*, and *cdrA* were PCR-amplified using high-fidelity Pfu DNA polymerase (Fermentas). The promoter regions were cloned into the BamHI–XhoI site upstream of the *lux/gfp* gene(s) on pMS402. Cloned promoter sequences were confirmed by DNA sequencing. Primers used are listed in Table 2. The reporter plasmid pKD-*cdrA,* pMS402-$sigX_{gfp}$, and pMS402-$oprF_{gfp}$ were transformed into *P. aeruginosa* respectively by electroporation. Besides the plasmid-based reporter system, an integration plasmid CTX6.1 originating from plasmid mini-CTX-*lux* (Becher and Schweizer 2000) was used to construct chromosomal fusion reporters for *exoS* and *rsmY*. This plasmid has all the elements required for integration, the origin of replication, and a tetracycline-resistance marker. The pMS402 fragment containing the kanamycin-resistance marker, the multiple cloning site (MCS), and the promoter-*luxCDABE* reporter cassette was then isolated and ligated into CTX6.1. The plasmid generated was first transferred into *E. coli* SM10λ-*pir* (Simon et al. 1983) and the *P. aeruginosa* reporter integration strain was obtained using bi-parental mating as reported previously (Hoang et al. 2000; Liang et al. 2008). These *lux* and *gfp*-based reporters were cultured overnight followed by dilution into fresh medium to an optical density at 600 nm (OD_{600}) of 0.2 and cultivated for an additional 3 h before use as inoculants. The cultures were inoculated into 96-well black (Fluorescence) or white (luminescence)

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
E. coli		
SM10-λ *pir*	Mobilizing strain, RP4 integrated in the chromosome; Kn^r	(Simon et al. 1983)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔM15 Tn10 (Tet^r)].*	Invitrogen
pRK2013	Broad-host-range helper vector*; Tra$^+$, Kn^r*	(Ditta et al. 1980)
P. aeruginosa		
PAO1	Wild type *P. aeruginosa*	This lab
PA (ΔPA1775)	PA1775 (*cmpX*) Knockout mutant of PAO1	This lab
Plasmids		
CTX6.1	Integration plasmid origins of plasmid mini-CTX-*lux*; Tc^r	This lab
pAK-1900	*E. coli-P. aeruginosa* shuttle cloning vector carrying p*lac* upstream of MCS; Ap^r,Cb^r	This lab
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE* gene; Kn^r,Tmp^r	(Duan et al. 2003)
$pMS402_{gfp}$	Expression reporter plasmid carrying the promoterless *gfp* gene; Kn^r,Tmp^r	This study
pEX18Tc	oriT$^+$ sacB$^+$ gene replacement vector with multiple-cloning site from pUC18; Tc^r	(Hoang et al. 1998)
pAK-1775	pAK1900 with a 1289 bp fragment of PA1775 between SphI and HindIII; Ap^r, Cb^r	This Lab
CTX-*exoS*	Integration plasmid, CTX6.1 with a fragment of pKD-*exoS* containing *exoS* promoter region and *luxCDABE* gene; Kn^r, Tmp^r, Tc^r	This Lab
CTX-*rsmY*	Integration plasmid, CTX6.1 with a fragment of pKD-*rsmY* containing *rsmY* promoter region and *luxCDABE* gene; Kn^r, Tmp^r, Tc^r	This lab
pKD-*cdrA*	The pMS402 plasmid containing the *cdrA* promoter region and *luxCDABE* reporter cassette; Kn^r, Tmp^r	This lab
pMS402-$sigX_{gfp}$	The pMS402 plasmid containing the *SigX* promoter region and *gfp r*eporter gene; Kn^r, Tmp^r	This lab
pMS402-$oprF_{gfp}$	The pMS402 plasmid containing the *oprF* promoter region and *gfp r*eporter gene; Kn^r, Tmp^r	This lab

plates with transparent bottom in triplicates in a ratio of 5 µL of inoculum to 95 µL of fresh medium. 50 µL of filter-sterilized mineral oil (Sigma Aldrich) was added on top to prevent evaporation during the assay. Both luminescence (counts per second, cps) and fluorescence from promoter-*gfp* reporter fusions (excitation wavelength of 490 nm and an emission of 510 nm) were measured every 30 min for 24 h in a Synergy H4 Multimode Microplate Reader (BioTek). Bacterial growth (OD_{600}) was measured simultaneously. The gene expression was proportional to level of light production. The level of gene expression was normalized to bacterial growth and is presented as relative luminescence units (RLU), calculated as cps/OD_{600} or relative fluorescence unit (RFU) calculated as RFU/OD_{600}. Strains carrying the empty vector $pMS402_{gfp}$ were measured for background levels corrections. All the data presented in this manuscript are means of three independent biological replicates.

Gene Knockout and Complementation

For construction of *cmpX* (PA1775) gene knockout mutant, fragment upstream to PA1775 was amplified using primer PA1775QS1 paired with PA1775QA1 and the downstream fragment was amplified using primer PA1775QS2 paired with PA1775QA2. The sequences of these primers are listed in Table 2. The two fragments were ligated into pEX18Tc vector to obtain the pEX18-PA1775. The *Ω* fragment obtained from pHP45*Ω* with streptomycin resistance gene was inserted in the middle of the two amplified PA1775 fragments. The resultant plasmid was transformed into PAO1 using the tri-parental mating procedure in which the strain carrying the helper plasmid pRK2013 was used together with the donor and recipient (Ditta et al. 1980). PAO1 *cmpX* mutants were then selected for resistance to streptomycin. The resulting mutant was verified by PCR. Here and elsewhere in the manuscript, *cmpX* refers to PA1775 as well as a *cmpX* knockout refers to ΔPA1775 and

Table 2 Primers used in this study

Primer	Sequence (5′–3′)
PA1775QS1-F	GCGGAATTCAACCGCACCATGTATCTC
PA1775QA1-R	TCAGGATCCGTTCACTCAGCCTTGTCA
PA1775QS2-F	TCAGGATCCCCGGGAGAGATAATGGAC
PA1775QA2-R	GCGAAGCTTTGACATGGACGAGCCAAC
pAK-PA1775-F	TAAGCATGCATCCGTTCGAAGAGTCCC
pAK-PA1775-R	GACAAGCTTAGACGTTGAACAGCGTGC
rpoD-F	CCTGATGAAGGCGGTGGAC
rpoD-R	GATGCGGATGGTGCGTGC
oprF-F	CAGTACCCGTCCACTTCCAC
oprF-R	TTCACGCGACCACCTTCTAC
sigX-F	AATTGATGCGGCGTTACCA
sigX-R	CCAGGTAGCGGGCACAGA
wspR-F	GCGGTCATGGTACTGCTTGT
wspR-R	CCGTCGGCTTGATCTGGTTG
pKD-*cdrA*-F	TTACTCGAGCTATCTGCGTGGCGCACGTCAG
pKD-*cdrA*-R	GTAGGATCCGGAAGGTTCCTTGGCGGCAGCG
pKD-*rsmY*-F	ATCTCGAGCAGTTCCTGGAGCTGGA
pKD-*rsmY*-R	GTCGGATCCTCTATCCTGACATCCG
pMS402-*sigX*-F	TTACTCGAGTCCTGGCGCAACTGGTGA
pMS402-*sigX*-R	GTAGGATCCGTGAGATCAGGCCAGTCAT
pMS402-*oprF*-F	TTACTCGAGGACGTGGCTGCTCTGCAGG
pMS402-*oprF*-R	GTAGGATCCCCGTTAAATCCCCATCTTG

F forward primer, *R* reverse primer

vice versa. ΔPA1775 in PAO1 (PA(ΔPA1775)) has been shown as ΔPA1775.

The multi-copy-number *E. coli–P. aeruginosa* shuttle vector pAK-1900 (Poole et al. 1993) was used for complementation of PA1775 into ΔPA1775 (ΔPA1775 (pAK-PA1775)). Full length PA1775 was PCR-amplified. The primers used are listed in Table 2. The PCR products were digested with *Sph*I and *Hin*dIII, and then cloned into pAK-1900 under control of a P*lac* promoter. Cloned PA1775 sequence was confirmed by DNA sequencing. The constructed plasmid was then transformed into *P. aeruginosa* by electroporation.

Metabolic Phenotype Array and Confirmation Assays

Metabolic phenotype arrays of *P. aeruginosa* strains PAO1 and ΔPA1775 were performed by commercially available, Biolog Phenotype MicroArrays (PM) (Biolog Inc., Harvard, CA, USA). Biolog PMs are 20, 96-well microtiter plates with each well containing defined medium with a unique metabolic substrate and tetrazolium dye as an indicator for cell respiration which is reduced by the action of dehydrogenases, yielding a purple formazan dye. PM1 to PM8 are metabolic panels containing various N, P, or S sources. Panels PM9 to PM20 measure sensitivity to salt, pH stress, antibiotics, anti-metabolites, and other inhibitors. Two replicates were conducted for each strain. Incubation and recording of phenotypic data were performed with an OmniLog instrument at 490 nm (Bochner 2009). For each strain, 1920 phenotypes were recorded simultaneously four times each hour by the OmniLog. A color change indicates that the cells are actively metabolizing a substrate in the well, while the lack of color change implies that the cells are unable to utilize the substrate.

To confirm the results of PM analysis, some of the key findings were subsequently investigated by performing independent assays. Thus, susceptibilities to antimicrobial agents were tested using broth microdilution method in Mueller–Hinton broth (Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom) according to the CLSI guidelines (CLSI 2012). Tested compounds: Orphenadrine (Cat # 75517), Compound 48/80 (Cat# C2313), Lauryl sulfobetaine (Cat # D0431), Methyltrioctylammonium chloride (Cat # 69485), Vancomycin (Cat # 1709007), Tobramycin (Cat # T4014), and Josamycin (Cat # 59983) were purchased from Sigma Aldrich Co. (St. Louis, Mo.). PAO1 and ΔPA1775 were cultured and the inoculum volume for minimum inhibitory concentration (MIC) testing was adjusted to a concentration of 5×10^5 CFU/ml. *P.*

aeruginosa PAO1 was used as the control strain in each run. The MIC was defined as the lowest antibiotic concentration that inhibited visible growth of the organism.

Bacterial Motility Assays

Bacteria were assessed for swarming, swimming, and twitching motility according to a protocol described previously (Rashid and Kornberg 2000). The swarming motility assay medium consisted of 0.5% agar, 8 g/L nutrient broth, and 5 g/L glucose. For the swimming motility assay, the medium consisted of 10 g/L tryptone, 5 g/L NaCl, and 0.3% agar. The medium for the twitching motility assay consisted of 4 g/L tryptone, 2 g/L yeast extract, 2 g/L NaCl, 1/L g $MgSO_4 \cdot 7H_2O$ solidified with 1% agar. For swimming and swarming motility assays, bacteria were spotted with 3 µL (2.0×10^8 cells/ml) of overnight LB cultures followed by incubation at 37 and 30 °C, respectively, for 12–14 h. Twitching motility was evaluated by seeding stationary-phase cells onto the centers of 100 mm diameter motility plates using a sterile toothpick. Plates were incubated at 37 °C. The diameters of the migration front were measured after 12–14 h of incubation. Photographs were taken with a Vilber Lourmat Fusion FX7 imager.

Measurement of Biofilm Formation

Biofilm formation was measured as described previously (O'Toole and Kolter 1998) with minor modifications. Cells from overnight cultures were inoculated at 1:100 dilutions in M9 medium supplemented with 0.2% glucose, 1 mM $MgSO_4$, and 0.5% casamino acids in 96-well polystyrene microtiter plates (Costar) and grown at 30 °C for 24 h. The planktonic fraction of cultures was removed from wells followed by three washes with phosphate buffered saline (PBS). A 100 µL crystal violet solution was added to each well, and staining was allowed for 15 min. The solution was then removed by aspiration. The wells were rinsed three times with distilled water, and the remaining crystal violet was dissolved in 125 µL of 30% acetic acid. Absorbance was measured at 590 nm.

Isolation of Total RNA

Strains were inoculated from glycerol stocks into 2 mL LB medium and grown overnight at 37 °C followed by subculturing in 5 mL LB medium and grown to mid-exponential phase. A 0.25-mL aliquot of the cell culture, corresponding to 5×10^8 cells, was added to 0.5 mL of RNeasy bacteria protect solution (Qiagen, Hilden, Germany). Total RNA was isolated according to the manufacturer's instructions. Residual DNA was eliminated by DNase treatment using 20 U of RQ1 (RNA Qualified) RNase-free DNase (Promega, Madison, WI). After removal of DNase, RNA was extracted and resuspended in 30 µL of RNase-free H_2O. The presence of residual DNA and the RNA quality was checked by formaldehyde/agarose gel electrophoresis.

Synthesis of cDNA and Quantitative Real-Time PCR (qPCR)

cDNA was synthesized from 500 ng of total RNA and a mixture of oligo dT and random primers using the Quanta qScript cDNA Synthesis kit (Quanta BioSciences, MD) as per the manufacturer's instructions. cDNA was stored at − 20 °C and unused RNA was stored at − 80 °C. As a quality control, RNAs were checked for the absence of genomic DNA contamination by quantitative real-time PCR with primers for the housekeeping gene *rpoD*.

qPCR was performed using SYBR select mastermix (Invitrogen) on an Eco illumina real-time detection system (Montreal Biotech). Reaction mixtures with a final volume of 20 µL consisted of 2 µL reverse transcribed cDNA, 5 pmol primers, 1x SYBR green containing dNTP mix and Taq polymerase. The reaction consisted of the following steps; an initial denaturation step of 2 min at 95 °C followed by 40 cycles of : 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 1 min. This was followed by the melt curve analysis. Melt curve analysis confirmed the presence of a single PCR product in each reaction. Gene expression fold-change was calculated using the ΔΔ Ct method. Ct values of each gene tested were normalized to the Ct values of the housekeeping gene *rpoD* (Park et al. 2013). Data are presented as percent change relative to gene expression in wild-type PAO1.

Statistical Analysis

Statistical analysis was performed using unpaired *t* test or one-way analysis of variance (ANOVA) with the Tukey or Dunnett post hoc test from a minimum of three independent experiments to determine the statistical significance when applicable: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; and $****p < 0.0001$.

Results

Role of *cmpX* (PA1775) in Virulence, Motility, and Biofilm Formation

We have previously demonstrated that PA1611 can bind to RetS and allows for activated transcription of RsmY and RsmZ and represses T3SS (Bhagirath et al. 2017; Kong

et al. 2013). The yet uncharacterized gene PA1775 (*cmpX*) has emerged as a regulator for PA1611 expression since a *cmpX* transposon insertion mutant showed activated expression of PA1611 (Kong et al. 2013). To understand the role of *cmpX*, we constructed a *cmpX* knockout mutant in PAO1 (ΔPA1775) as well as a complementation strain, ΔPA1775 (pAK-PA1775). We examined the effect of *cmpX* deletion on T3SS effector *exoS* and on small RNA RsmY (Fig. 1). As shown in Fig. 1a, the *exoS* promoter activity in the *cmpX* knockout strain was at least twofold lower than in PAO1 under T3SS inducing conditions. The *exoS* expression level in the mutant was reverted to the wild-type level upon complementation. Similarly, the effect of the *cmpX* on biofilm formation was tested. As seen in Fig. 1b, ΔPA1775 formed 1.5-fold higher biofilm than its wild-type control ($p < 0.01$). This was reversible to wild-type levels in ΔPA1775 (pAK-PA1775). Biofilm formation in *Pseudomonas* involves tight control over the transcription of the regulatory small RNAs, *rsmY*, and *rsmZ*. The impact of the *cmpX* knockout in PAO1 on levels of RsmY and RsmZ was further measured using the *lux*-based reporters. Here, RsmY was used a representative. As shown in Fig. 1c, a fourfold higher *rsmY* promoter activity was observed in ΔPA1775 as compared with wild-type PAO1. This was also reversible upon complementation with PA1775 on plasmid.

Acute phase of *P. aeruginosa* is characterized by high motility and is regulated by GacA/S TCS (Li et al. 2017). RsmA also regulates *exoS* expression and positively regulates motility (Heurlier et al. 2004). Thus, to further analyze the phenotypical effect of *cmpX* on motility, ΔPA1775 was analyzed for swimming, swarming, and twitching motility (Fig. 2). These motilities were assessed by measuring the diameters of the migration front after 12–14 h of incubation. As compared to wild-type PAO1, ΔPA1775 demonstrated reduced swarming ($p < 0.01$) and twitching ($p < 0.001$) motility as shown in Fig. 2a and c. No significant difference in swimming motility was visible (Fig. 2b).

CmpX is Involved in Signaling via c-di-GMP

Though our results for the role of *cmpX* in virulence and biofilm formation were in alignment with the PA1611-RetS-GacS/GacA-RsmA/Y/Z pathway and could be explained as such, an alternative pathway that has been increasingly shown to regulate virulence and bacterial phenotypes involves signaling by c-di-GMP. Thus, to confirm if the observed phenotypes could be a result of altered c-di-GMP levels, we tested *cdrA* promoter activity within a *cmpX* knockout and wild-type PAO1. Previously, *cdrA* promoter activity levels have been shown to faithfully reflect the fluctuations in intracellular c-di-GMP levels (Bouffartigues et al. 2015; Rybtke et al. 2012); hence, we measured *cdrA* levels to indirectly quantify c-di-GMP levels in ΔPA1775. Quantifications were performed in triplicates and the data at 12 h are presented in Fig. 3a as relative luminescence units (RLUs). To avoid conditional changes in *cdrA* levels, the growth conditions for PAO1 and ΔPA1775 were kept the same. ΔPA1775 demonstrated twofold higher levels of *cdrA* promoter activity than in the wild-type PAO1 ($p < 0.001$), indicating a clear effect of *cmpX* on intracellular c-di-GMP levels.

A chemosensory-like surface-sensing system *wspR* encodes c-di-GMP synthase (Guvener and Harwood 2007), and has been shown to be involved in modulation of c-di-GMP levels and switch between T3SS and T6SS (Moscoso et al. 2011) by control of Pel synthesis. Thus, we wanted to examine if the observed increase in c-di-GMP levels could be attributable to *wspR* activation. We examined the transcript levels of *wspR* in the *cmpX* knockout mutant. As seen in Fig. 3b, the *cmpX* knockout mutant demonstrates 1.7-fold higher expression as compared to that in the wild-type PAO1, suggesting that the increased c-di-GMP levels in the *cmpX* knockout mutant may have at least partially resulted from the activated *wspR* expression.

The Extracytoplasmic Function Sigma Factor *sigX* and Outer Membrane Porin, *oprF* are Down-Regulated in *cmpX* Knockout Mutant

On the *P. aeruginosa* genome, *cmpX* is located immediately upstream of *sigX* and *oprF* genes. Outer membrane porin OprF has been shown to be involved in cell survival and in maintaining cell wall integrity (Rawling et al. 1998; Woodruff and Hancock 1989). Extracytoplasmic function sigma factor *sigX* has also been shown to be involved in virulence regulation, fatty acid biosynthesis, and maintaining membrane homeostasis (Brinkman et al. 1999). Both *oprF* and sigX knockout mutants demonstrated downregulation of *cmpX*, activation of small RNAs, and elevated c-di-GMP levels by activation of diguanylate cyclase adcA (PA4843) and PA1181 (Bouffartigues et al. 2012, 2015; Brinkman et al. 1999). Based on the genomic location, we wondered if *cmpX* had an effect on *sigX* and *oprF* which could contribute to the elevation in c-di-GMP levels. Thus, the effect of *cmpX* knockout on *sigX* and *oprF* was investigated by using qPCR. The expression levels of *oprF* and *sigX* in ΔPA1775 were compared with those in wild-type PAO1. As seen in Fig. 4, the expression of *sigX* and *oprF* (Fig. 4a) was decreased by threefold and 2.3-fold respectively in the *cmpX* mutant. We further confirmed this result by measuring promoter activity of *sigX* and *oprF* in the ΔPA1775 strain, and similar results were obtained (Fig. 4b, c). These results suggest that *cmpX* affects *sigX* and *oprF,* which could consequently influence intracellular

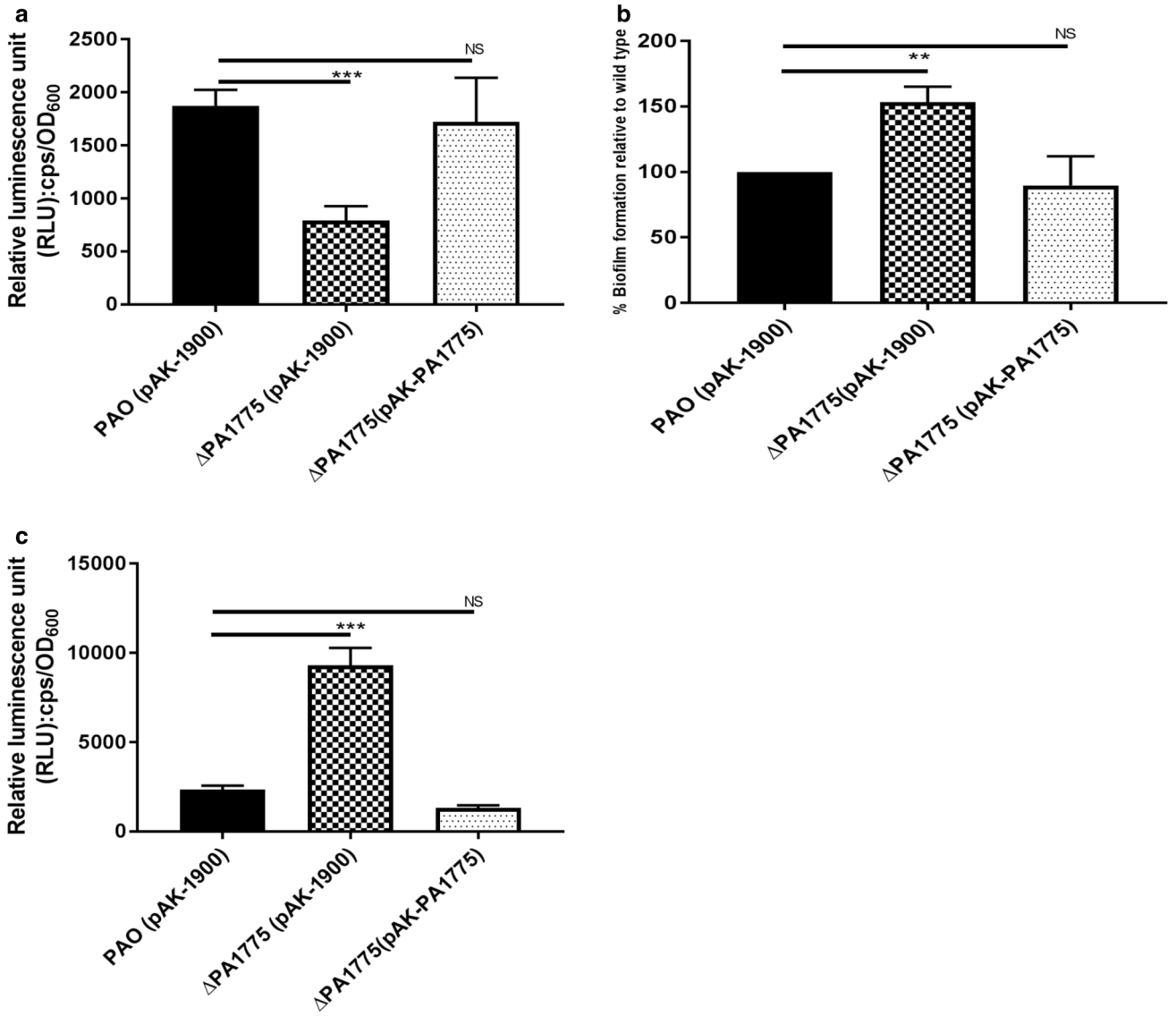

Fig. 1 Effect of a *cmpX* knockout on virulence and biofilm formation. **a** *exoS* promoter activity at 12 h. **b** Biofilm formation of the ΔPA1775 and a ΔPA1775 (pAK-PA1775) complementation strain as compared with that of the wild-type PAO1. Data are shown as the percent change relative to PAO1. **c** *rsmY* promoter activity at 12 h. A CTX-*exoS* and CTX-*rsmY* reporter fusion integrated to the chromosome was used to measure the *exoS* and *rsmY* promoter activity, respectively. CTX-*exoS* promoter activity was measured under T3SS inducing conditions. These experiments were done in wild type PAO1, ΔPA1775, and ΔPA1775 background containing the expression plasmid pAK-PA1775 carrying the full length PA1775 in the expression vector pAK-1900 (ΔPA1775 (pAK-PA1775)). PAO1 and ΔPA1775 contain empty vector pAK-1900. PAO1 is used as the control. The *exoS* promoter activity at 12 h is normalized to growth and shown in relative luminescence unit (RLU): cps (counts per second)/OD_{600}. The values shown are average of three independent experiments. Data were analyzed using one-way ANOVA with Dunnett post hoc test. NS $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Error bars indicate standard deviations

c-di-GMP levels and affect virulence factors in *P. aeruginosa*.

PA1775 Promoter Activity During Growth in PAO1, Biolog Phenotype MicroArrays and Validation

PA1775 has been annotated as a putative conserved cytoplasmic membrane protein in the *Pseudomonas* genome database (Winsor et al. 2016). To investigate its potential roles, the expression profile of *cmpX* and the metabolic profiles of *cmpX* knockout mutant were examined using PA1775 expression reporter and Biolog phenotype microarrays, respectively.

The CTX–PA1775 reporter fusion was integrated into the chromosome of PAO1, and PA1775 promoter activity was monitored over an extended period of growth. Figure 5

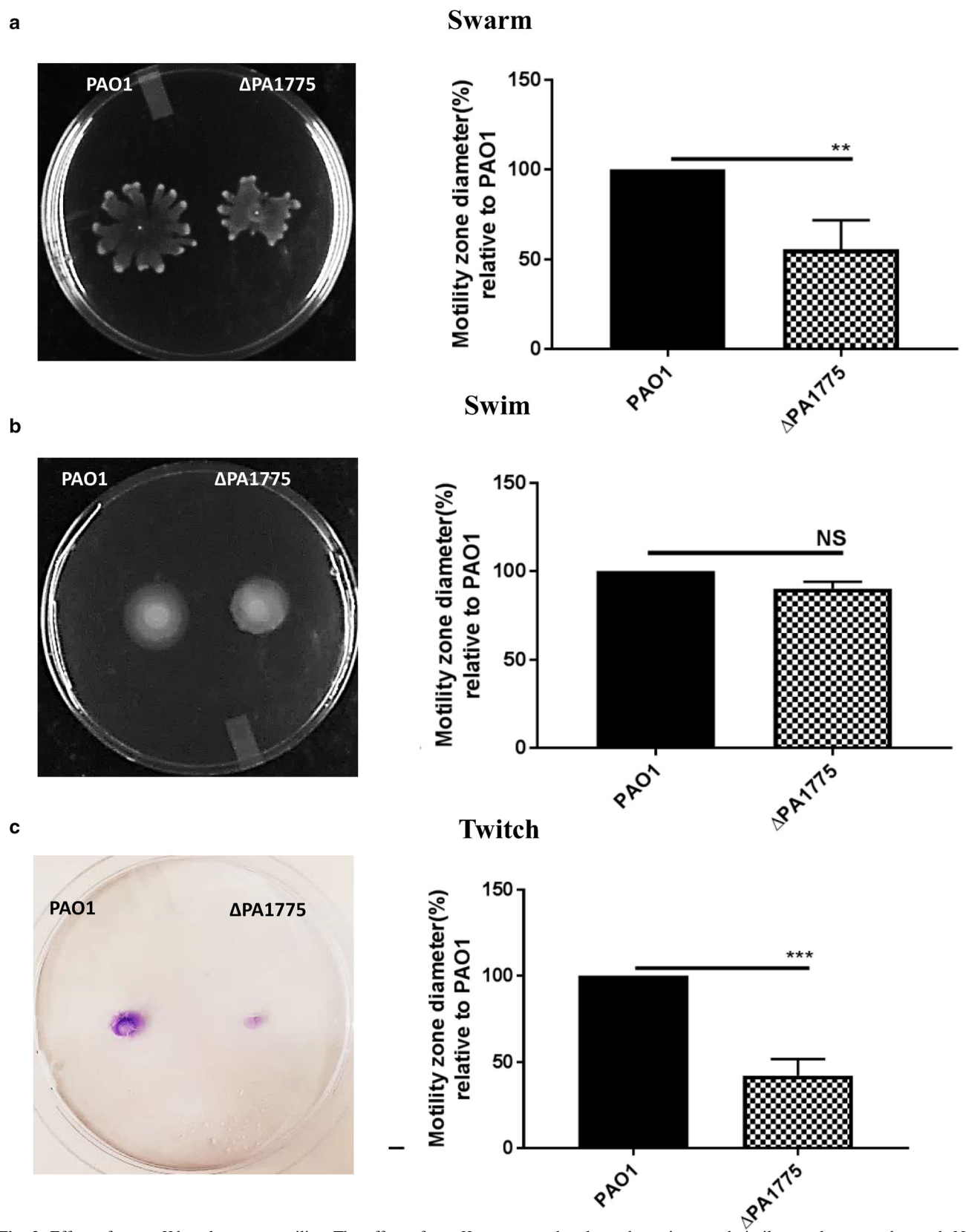

Fig. 2 Effect of a *cmpX* knockout on motility. The effect of *cmpX* knockout on swarming (**a**), swimming (**b**), and twitching (**c**) motility are shown. Diameter of the zone of motility are expressed in percentages relative to the wild-type PAO1. The experiments were repeated at least three times and similar results were observed. NS $p > 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars indicate standard deviations

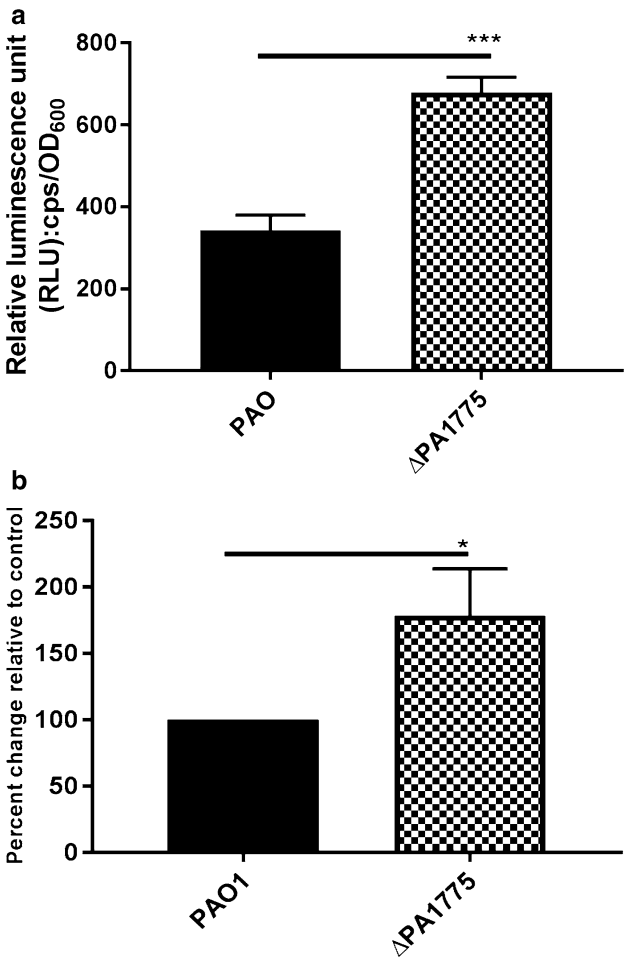

Fig. 3 CmpX is involved in signaling via c-di-GMP. **a** *cdrA* promoter activity in PAO1 and ΔPA1775. The promoter region for *cdrA* was cloned into the pMS402 upstream of the *lux* genes. The resultant plasmid pKD-*cdrA* was transformed into *P. aeruginosa* respectively by electroporation. The wild-type PAO1 is used as the control. *cdrA* promoter activity at 12 h is normalized to growth and shown in relative luminescence unit (RLU): cps (counts per second)/OD_{600}. **b** mRNA expression of *wspR* was normalized to the housekeeping gene *rpoD* in PAO1 and ΔPA1775. Data are presented as percent change relative to PAO1. The values shown are average of three independent experiments. Data were analyzed using unpaired *t* test. The error bars indicate standard deviations. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

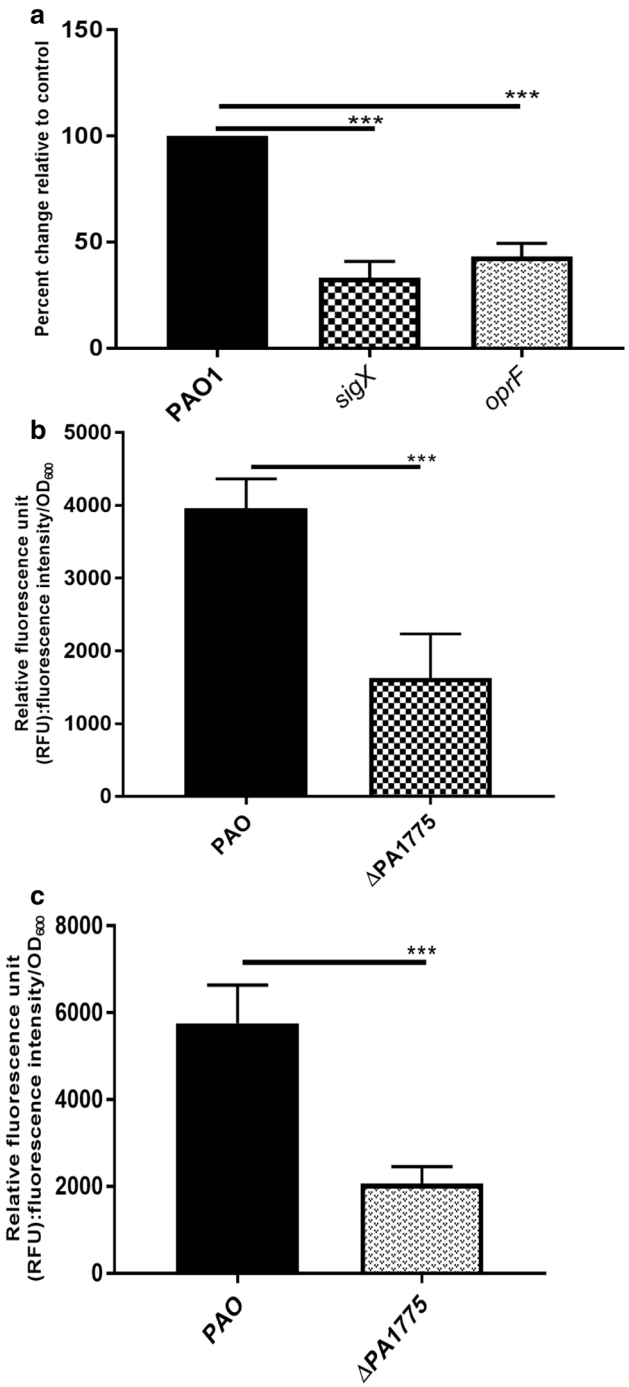

Fig. 4 Effect of a *cmpX* knockout on *sigX* and *oprF* gene expression. **a** mRNA expression for *sigX* and oprF were normalized to the housekeeping gene *rpoD* in PAO1 and ΔPA1775, respectively. Data are presented as percent change relative to PAO1. *sigX* (**b**) and oprF (**c**) promoter activity in PAO1 and ΔPA1775. The promoter region for *sigX* and *oprF* was cloned into the pMS402 upstream of the *gfp* gene. The resultant plasmid pMS402-$sigX_{gfp}$ and pMS402-$oprF_{gfp}$ were transformed into *P. aeruginosa* respectively by electroporation. The wild-type PAO1 is used as the control. *sigX* and *oprF* promoter activity at 12 h is normalized to growth and shown in relative fluorescence unit (RFU) calculated as RFU/OD_{600}. Strains carrying the empty vector $pMS402_{gfp}$ were measured for background levels corrections. Data were analyzed using unpaired *t* test. $**p < 0.01$ and $***p < 0.001$

shows PA1775 promoter activity over 70 h with growth monitored simultaneously. PA1775 demonstrated an increased promoter activity during the early log phase of growth followed by a second surge during late stationary phase, suggesting that it responds to signals present in these growth phases and may be cell density dependent.

Biolog phenotype microarrays have been used to facilitate the characterization of unknown genes (Johnson et al. 2008). Despite the preliminary experiments showing a significant role of *cmpX* in controlling phenotypes in *P. aeruginosa*, the function of CmpX has been relatively

unexplored. Hence, we used PM technology to study the potential function of CmpX in *P. aeruginosa*. Table 3 outlines the phenotypes lost and gained in a PA1775 knockout strain. To confirm the results obtained through the PMs some of the key findings were subsequently verified (Table 4). The results confirmed that the *cmpX* mutants demonstrated greater sensitivity to membrane detergents such as lauryl sulfobetaine, environmental toxins such as potassium tellurite, cell wall synthesis inhibitor, and protein synthesis inhibitors such as tobramycin. Highest difference in sensitivity for the *cmpX* mutant was observed for tobramycin (0.3 µg/ml), vancomycin (150 µg/ml), potassium tellurite (4 µg/ml), and methyltrioctylammonium chloride (3 µg/ml).

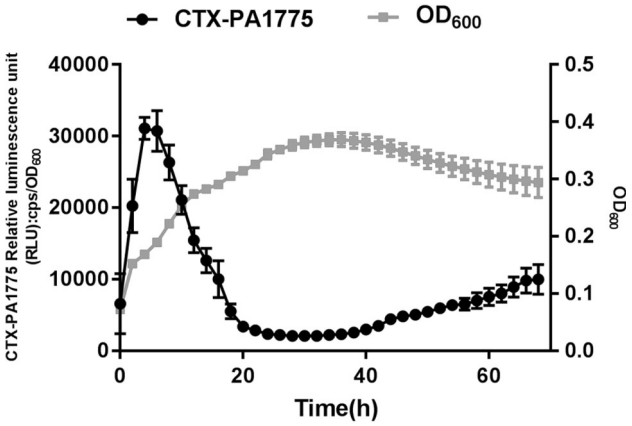

Fig. 5 PA1775 promoter activity during growth in PAO1 over time. A CTX-PA1775 reporter fusion integrated to the chromosome was used to measure the PA1775 promoter activity (solid line). Bacterial growth was monitored simultaneously and (OD_{600}) is shown in gray-dotted line. The PA1775 promoter activity is normalized to growth and shown as relative luminescence unit (RLU): cps (counts per second)/OD_{600}. The values shown are average of three independent experiments. The error bars indicate standard deviations

Discussion

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium, capable of growing in various conditions, even in distilled water (Favero et al. 1971). Its adaptability is attributable to the complex and extensive gene regulation, as well as intricate signaling mechanisms.

Previously, we have shown that a *cmpX* transposon mutant affected the expression of PA1611 (Kong et al. 2013). PA1611 is a HHK, which senses yet unknown environmental cues to regulate biofilm formation.

Table 3 Summary of gained and lost phenotypes for *P. aeruginosa cmpX* knockout mutants

Index	Phenotypes lost	Metabolic values	Function
1	Orphenadrine	− 146	Anti-cholinergic
2	D,L-Propanolol	− 113	Beta-adrenergic blocker
3	Compound 48/80	− 101	Cyclic AMP phosphodiesterase inhibitor
4	Methyltrioctylammonium chloride	− 106	Membrane, detergent, cationic
5	Dodecyltrimethyl ammonium bromide	− 74	Membrane, detergent, cationic
6	N-Dodecyl-N,N-dimethyl-3- ammonio-1-propanesulfonate) Lauryl sulfobetaine	− 287	Membrane, detergent, zwitterionic
7	Lysine-Tryptophan	− 94	N-Source, peptide
8	L-Asparagine	− 53	Nutritional supplement
9	Blasticidin S	− 194	Protein synthesis
10	Tobramycin	− 221	Protein synthesis, 30S ribosomal subunit, aminoglycoside
11	Josamycin	− 136	Protein synthesis, 50S ribosomal subunit, macrolide
12	D,L-Thioctic acid	− 250	Reducing agent
13	Potassium tellurite	− 742	Toxic anion
14	Vancomycin	− 87	Cell wall synthesis inhibitor
Phenotypes gained			
15	L-Aspartic acid	67	C-Source, amino acid
16	L-Glutamic acid	54	C-Source, amino acid

Table shows the phenotypes gained or lost by *cmpX* mutants as compared to wild-type PAO1. Phenotype microarrays were performed by the commercially available Biolog PM and data were analyzed by the OmniLog® V. 1.5 comparison module and the average height parameters were used for data analysis with standard thresholds for detection. The metabolic distance threshold was set at 50 and the sensitivity threshold was set at 74

Table 4 Confirmed phenotypes for *cmpX*

Compound	PAO1	ΔPA1775
Tobramycin	2 μg/ml	0.3 μg/ml
Vancomycin	300 μg/ml	150 μg/ml
Orphendrine	800 μg/ml	600 μg/ml
Compound 48/80	10 μg/ml	8 μg/ml
Lauryl sulfobetaine	3 mg/ml	2.5 mg/ml
Methyltrioctylammonium chloride	6 μg/ml	3 μg/ml
Potassium tellurite	10 μg/ml	4 μg/ml

Table shows MIC for *cmpX* knockout (ΔPA1775) and its wild-type control PAO1. MIC was defined as the lowest antibiotic concentration that inhibited visible growth of the organism

A PA1611 knockout in PAO1 demonstrated increased T3SS effector secretion and decreased biofilm formation by direct interaction with RetS (Bhagirath et al. 2017). However, how *cmpX* affects PA1611 and the role of *cmpX* in the signal transduction network remained unclear.

Here, we confirmed that *cmpX* plays an important role in PA1611-RetS-GacS/GacA-RsmA/Y/Z pathway in *P. aeruginosa.* The *cmpX* knockout mutant demonstrates decreased expression of T3SS effector *exoS* and increased expression of *rsmY*, as well as enhanced biofilm formation. These results are in agreement with the previously proposed role of *cmpX* in affecting PA1611 (Kong et al. 2013). We also demonstrate that the effect of *cmpX* on virulence and biofilm formation could be a combined result of elevated c-di-GMP and its effect on the *sigX* and *oprF* genes.

Expression of *cdrA* encoding for an adhesin in *P. aeruginosa* was previously shown to be highly co-relatable to changes in the levels of intracellular c-di-GMP (Rybtke et al. 2012). As compared to PAO1, the *cmpX* knockout demonstrated an increase in *cdrA* promoter activity, suggesting elevated c-di-GMP levels in the mutant. High levels of c-di-GMP have been shown to affect T3SS negatively and increase polysaccharide production by activation of *pel* system and thus enhance biofilm formation (Borlee et al. 2010; Guvener and Harwood 2007; Hickman et al. 2005; Moscoso et al. 2011; Starkey et al. 2009).

We further examined *wspR,* a c-di-GMP synthase associated with the RetS/GacS signaling cascade (Moscoso et al. 2011). We hypothesized that this may explain the elevated c-di-GMP levels in ΔPA1775. *wspR* when overexpressed caused an increase in biofilm formation by activation of *pel* genes, whereas *wspR* mutant demonstrated loss of cytotoxicity and reduced biofilm formation (Kulasakara et al. 2006). Earlier, it has been shown that a *retS* knockout mutant demonstrated elevated c-di-GMP levels by activation of *wspR* (Moscoso et al. 2011). We have previously shown that the phenotype of a PA1611 overexpression strain was similar to that of *retS* knockout (Kong et al. 2013).

We have also shown that a *cmpX* transposon mutant activated PA1611. An activated PA1611 can bind to RetS (Bhagirath et al. 2017) and lead to elevated exopolysaccharide production and biofilm formation (Kong et al. 2013). Exopolysaccharide production in *P. aeruginosa* involves activation of *pel* genes and both RetS and c-di-GMP have been shown to converge on Pel regulon. In this study, we observed that the *cmpX* knockout demonstrates *wspR* activation, reduced T3SS effector and activating small RNA *rsmY* and increased biofilm formation. Taken together, these findings suggest that *cmpX* is connected to both GacS/A-RsmA/Y/Z as well as c-di-GMP signaling.

Motility assays demonstrated that only swarming and twitching motility were affected in the *cmpX* knockout mutant, whereas swimming motility did not exhibit any difference. The reduction of swarming but not swimming is intriguing, yet consistent with a previous observation (Zheng et al. 2016). The difference may lie in differential regulation for these two motility patterns. Swimming and swarming do share some common features, but are remarkably different in other aspects. Swarming is a group behavior that involves rapid and coordinated group movement across a hydrated semi-solid surface often typified by solar flare appearances. Swimming motility on the other hand is a mode of individual bacterial movement in liquid environment (< 0.3% agar) powered by rotating flagella. The effect of *cmpX* on swarming but not swimming may underline its role in regulating bacterial group behavior.

Our results also indicate that the transcription of *oprF* and *sigX* was significantly decreased in the *cmpX* mutant. *cmpX* is the last gene in the *cmaX-crfX-cmpX* operon and is located upstream of the sigma factor *sigX* and the major outer membrane porin gene *oprF* on the *P. aeruginosa* genome. *cmpX* encodes a AlgU binding site, a *sigX* consensus sequence in its promoter region as well as one of the promoters for *oprF* in the *cmpX-sigX* intergenic region. The other promoters for *oprF* have been identified in *sigX-oprF* intergenic region (controls > 80% transcription) and in *sigX* itself (Bouffartigues et al. 2012; Brinkman et al. 1999). Previous studies have shown that a *sigX* mutant demonstrated reduced transcription of *oprF*, reduced virulence, motility, and enhanced biofilm formation (Gicquel et al. 2013). OprF is involved in rhamnolipid production (Fito-Boncompte et al. 2011), biofilm development and resistance to antibiotics such as cephalosporins (Bratu et al. 2007). An *oprF* mutant demonstrated elevated levels of c-di-GMP (Bouffartigues et al. 2015), and reduction in T3SS effectors (ExoT and ExoS) as well as decreased production of pyocyanin, lectin PA-1, exotoxin A (Fito-

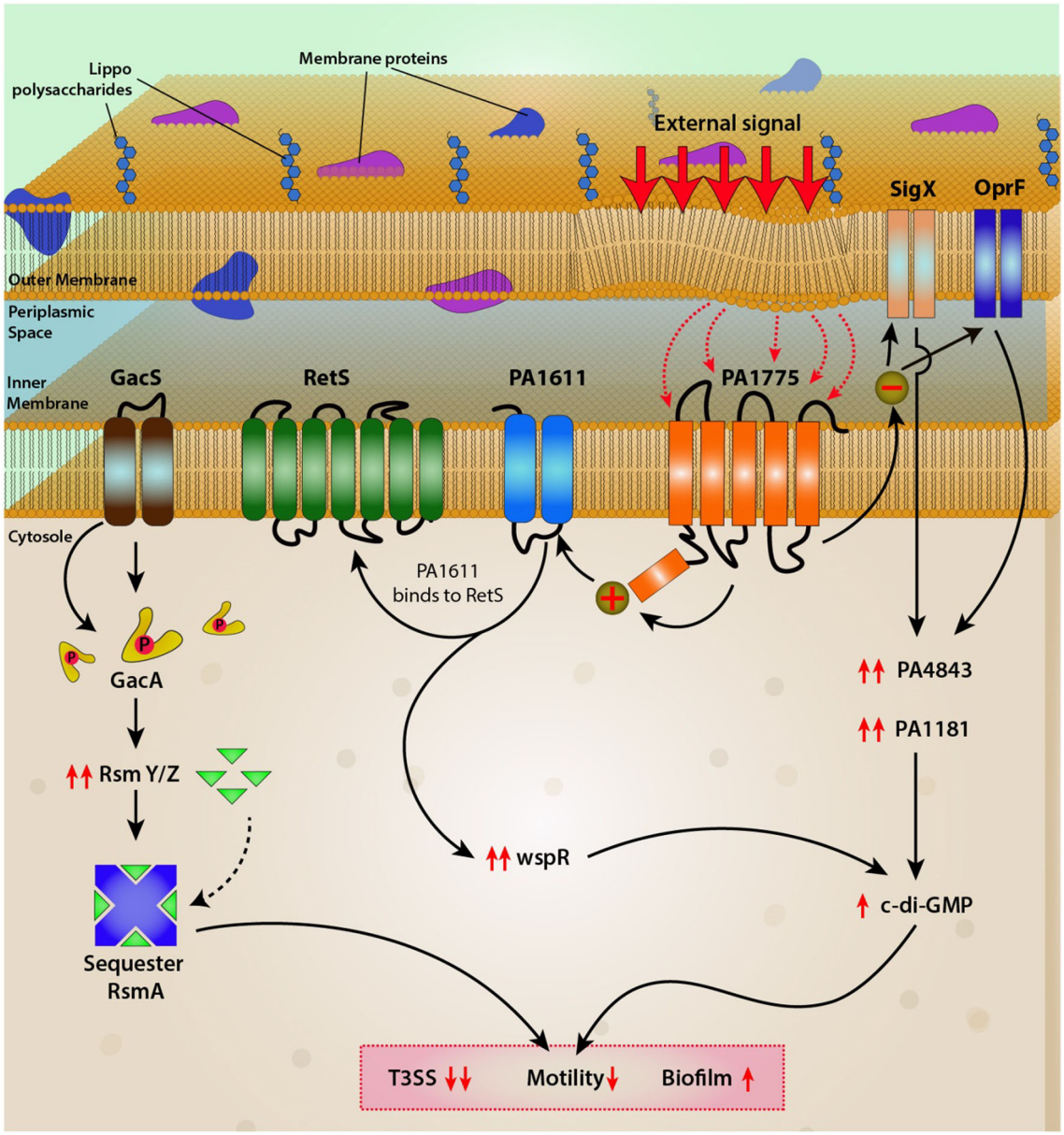

Fig. 6 Proposed role for *cmpX* in c-di-GMP regulation and PA1611-RetS-GacS/A-RsmA/Y/Z pathway. CmpX (PA1775), a putative small mechanosensor, senses changes in bacterial membrane. In response to external signals such as those in chronic infections (e.g., increased cell density), *cmpX* has been shown to activate PA1611 (Kong et al. 2013). Activated PA1611 has been shown to bind to RetS (Bhagirath et al. 2017). RetS mediates acute phase of infection by binding to GacS. Once RetS is unavailable, GacS is able to phosphorylate response regulator GacA and activate transcription of small RNAs, RsmY/RsmZ which sequester post-transcriptional regulator RsmA. It has been shown that RetS mutant activated c-di-GMP synthase WspR (Moscoso et al. 2011), which in turn increases intracellular levels of c-di-GMP. A *cmpX* mutant demonstrates decreased activity of *sigX* and *oprF*. An *oprF* and *sigX* mutant has been shown to activate c-di-GMP synthases PA4853 and PA1181 further contributing to increased c-di-GMP levels (Bouffartigues et al. 2015). All these factors together enhance biofilm formation, downregulate T3SS and motility. The cartoon is for representation only and is not drawn to scale

Boncompte et al. 2011). An *oprF* knockout resulted in activation of *pel* genes as well as PA4843 and PA1181 genes of the *sigX* regulon both of which encode c-di-GMP synthase (Bouffartigues et al. 2015). Considering the complex relationship between intracellular c-di-GMP levels and several other regulatory networks including the GacS/GacA-RsmA/Y/Z (Moscoso et al. 2011) and OprF (Bouffartigues et al. 2015) pathways, the effect of *cmpX* knockout on c-di-GMP levels could be a combined one (Fig. 6), although the exact role of *cmpX* in *oprF* regulation needs further investigation.

Upon comparing the metabolic and sensitivity capabilities of a *cmpX* mutant to wild-type PAO1, *cmpX* knockout demonstrated increased sensitivity to membrane detergents

such as lauryl sulfobetaine, and antibiotics such as vancomycin and tobramycin, suggesting that *cmpX* may play a role in the cell membrane function or structure. Interestingly, *cmpX* knockout also demonstrated an increased sensitivity to potassium tellurite an environmental toxin. Tellurite in the environment is a result of metalloid pollution. It is highly toxic to most bacteria and was understood in fact as an antimicrobial agent (Fleming and Young 1940). It was recently shown that tellurite is capable of inducing c-di-GMP levels in *P. aeruginosa.* When c-di-GMP levels were artificially reduced, *P. aeruginosa* demonstrated increased sensitivity to this toxic metalloid (Chua et al. 2015). Collectively, our findings of affected phenotypes in a *cmpX* mutant support previous studies and suggest that *cmpX* may have a more complex function. Further studies are needed to elucidate its roles in complete details.

Pseudomonas aeruginosa colonizes and grows in the host in form of biofilms by undergoing specific transition from planktonic to sessile state. This process involves sensing its environment and regulation of virulence factors. The transition of *Pseudomonas* from planktonic to attached cells during infection is profoundly complex. Bacteria in biofilms are physiologically very different from their planktonic counterparts, including antimicrobial resistance profiles (Bhagirath et al. 2016; Dubois-Brissonnet et al. 2016), but the developmental process remains to be understood. C-di-GMP levels in planktonic bacteria (< 30 pmol) are much lesser than those in biofilms (75-110 pmol) (Basu Roy and Sauer 2014). During early log phase when c-di-GMP levels are low the transcription of *cmpX* is much higher, whereas during stationary phase, the c-di-GMP levels increase and *cmpX* transcription declines. After biofilm matures, *P. aeruginosa* cells are released in a controlled manner back to a planktonic mode of growth. For this, a well-established nitrosative stress model has been postulated (Webb et al. 2003; Yoon et al. 2002). Upon addition of Sodium Nitroprusside (SNP) to biofilms, intracellular c-di-GMP levels in cells were shown to be decreased by 45 to 47% (Barraud et al. 2009) and resulted in dispersal of cells. Thus, a biofilm dispersion model may explain the activation of *cmpX* in the stationary phase.

cmpX falls in the category of highly conserved small membrane protein of 274 amino acids (aa). A domain analysis of *cmpX* shows a conserved, mechanosensor of small conductance (MscS) domain. Further studies are needed to define the exact mechanism by which *cmpX* participates in virulence regulation in *P. aeruginosa* and its potential role as a mechanosensor or other sensory component in the signal transduction pathway. Investigation of *cmpX* may not only help in our understanding of *P. aeruginosa* infection but also the function of such a class of membrane proteins.

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