Canadian Journal of Microbiology
Revue canadienne de de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2017-0244.R1
Manuscript Type:	Article
Date Submitted by the Author:	08-Jun-2017
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Keyword:	phospholipase, biofilm formation, hyperbiofilm, matrix, <i>Pseudomonas aeruginosa</i> ,

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Hyperbiofilm phenotype of *Pseudomonas aeruginosa* defective for the PlcB and PlcN secreted phospholipases.

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Abstract

Biofilms are dense communities of bacteria enmeshed in a protective extracellular matrix composed mainly of exopolysaccharides (EPS), extracellular DNA (eDNA), proteins and outer membrane vesicles (OMV). Given the role of biofilms in antibiotic tolerant and chronic infections, novel strategies are needed to block, disperse or degrade biofilms. Enzymes that degrade the biofilm matrix are a promising new therapy. We screened mutants in many of the enzymes secreted by the type II secretion system (T2SS) and determined that the T2SS, and specifically phospholipases, play a role in biofilm formation. Mutations in the *xcp* secretion system, and the *plcB* and *plcN* phospholipases all resulted in hyperbiofilm phenotypes. PlcB has activity against many phospholipids including the common bacterial membrane lipid phosphatidylethanolamine (PE), and may degrade cell membrane debris or OMVs in the biofilm matrix. Exogenous phospholipase was shown to reduce aggregation and biofilm formation, suggesting their potential role as a novel enzymatic treatment to dissolve biofilms.

Keywords: phospholipase, biofilm formation, hyperbiofilm, matrix, *Pseudomonas aeruginosa*, PlcB, PlcN,

Introduction

Biofilms are dense aggregates of microbial communities that contribute to chronic bacterial infections and numerous industrial fouling problems (Mattila, Sandholm and Wirtanen 1992; Davey and O'Toole 2000). Insights into the nature and mechanisms of biofilm formation have led to new treatment ideas about how to target biofilms. A hallmark feature of biofilms is the production of an extracellular matrix that promotes cell-to-cell and cell-to-surface interactions, leading to aggregation (Flemming and Wingender 2010). In addition to the important structural function of the biofilm matrix, other non-structural functions have been described. The biofilm matrix polymers play a role in shaping the microscale conditions of biofilms by sequestering divalent metal cations or acidifying biofilms (Mulcahy et al. 2008; Horsman et al. 2012; Wilton et al. 2015). In addition, the matrix polymers serve as nutrients available for recycling, contribute to the antibiotic resistance phenotype, to signaling, bacterial migration and the development of complex structures (Flemming and Wingender 2010; Lewenza 2013, Wilton et al. 2015; Dragoš and Kovács 2017).

The *P. aeruginosa* biofilm matrix is highly hydrated and composed primarily of exopolysaccharides and extracellular DNA (Flemming and Wingender 2010). The *P. aeruginosa* matrix also includes numerous proteins, such as lectins, the cross-linking CdrA protein, and the motility appendages flagella and type IV pili (O'Toole and Kolter 1998; Flemming and Wingender 2010; Gunn et al. 2016). Less focus has been placed on the lipid content of the matrix, but outer membrane vesicles (OMV) are an abundant component of the biofilm matrix (Schooling and Beveridge 2006; Gunn et al. 2016).

OMVs consist of phospholipids, lipopolysaccharide, membrane-bound and cargo proteins, and are released from the outer membrane.

Given the universal importance of the biofilm matrix, one important strategy aimed at breaking up biofilms is the use of enzymes to degrade the extracellular matrix (Flemming and Wingender 2010; Bernardes et al. 2015). Numerous enzymes have been employed to degrade components of the biofilm matrix, including deoxyribonucleases and restriction endonucleases that degrade DNA (Tetz et al. 2009; Kaplan 2014), glycoside hydrolases that degrade EPS (Fleming et al. 2016) such as PelA and PslG that degrade the Pel and Psl (Baker et al. 2016), and proteases to target matrix proteins (Kaplan 2014). Some quorum sensing (QS) quenching enzymes are able to disrupt biofilm formation by blocking QS signaling in biofilms on urinary catheters (Ivanova et al. 2015).

The purpose of this study was to examine the potential contribution of enzymes secreted by the *P. aeruginosa* type II secretion system (T2SS) to biofilm formation. The T2SS is a complex structure that permits the secretion of numerous enzymes to the outside of the cell, including proteases, lipases, phospholipase, deoxyribonuclease, phosphatase, among others (Mulcahy et al. 2010; Bleves et al. 2010). We report that mutants in the T2SS have a hyperbiofilm phenotype, which suggests that some type II secreted enzymes are contributing to biofilm development via degradation of the biofilm matrix. Individual knockouts of specific T2SS enzymes were also tested and revealed that phospholipase mutants shared the hyperbiofilm phenotype of the secretion system mutants. We demonstrate a role of secreted phospholipases in aggregation and biofilm

formation, and suggest a novel application for phospholipases for dissolving bacterial biofilms.

Materials and Methods.

Bacterial strains and growth media. The strains and plasmids used in this study are listed in Table 1. Planktonic cultures and biofilms were grown in Basal Minimal Medium 2 (BM2) at 37°C, with limiting magnesium and varying phosphate concentrations. BM2 media prepared with 20 mM sodium succinate, 100 mM Hepes pH 7.0, 7mM (NH₄)₂SO₄, 200 µM or 1.6 mM phosphate buffer pH 7.2, 100 µM MgSO₄, 10 µM FeSO₄ and ion solution, containing 1.6 mM MnSO₄·H₂O, 14 mM ZnCl₂, 4.7 mM H₃BO₃ and 0.7 mM CoCl₂·6H₂O. Limiting magnesium was used to promote EPS production and biofilm formation (Mulcahy and Lewenza 2011) and phosphate levels were adjusted to increase (200 µM) or reduce (1.6 mM) expression of the phospholipases. Exogenous phospholipase D from *Streptomyces chromofuscus* (Sigma P0065-25KU) was added as described.

Cloning and purification of PlcB. The *plcB* gene was amplified with primers F-PlcB and R-6xHIS-PlcB (Table 1) and cloned as a His-tagged PlcB (*EcoRI-HindIII*) into vector pPSV37 under the control of an IPTG inducible promoter. The ligation reaction was transformed directly into electrocompetent *P. aeruginosa*, as expression of foreign phospholipase in *Escherichia coli* was not tolerated, likely because the enzyme was lethal if not secreted. DNA sequencing was performed to confirm the correct *plcB* (PA0026) sequence.

P. aeruginosa expressing pPlcB was grown to mid-log in LB containing 30 µg/mL gentamicin, where 0.1 mM IPTG was added for an additional 3 hrs incubation. Cell pellets were collected, resuspended in wash buffer (0.5x PBS pH 7.1, 300 mM NaCl, 5 mM Imidazole), lysed by sonication (15 min; 5 sec on, 10 sec off) and centrifuged (15,000 RPM, 30 min). The soluble fraction was passed over a slurry of HisPur cobalt sepharose (ThermoFisher Scientific), eluted in elution buffer (0.5 PBS pH 7.1, 300 mM NaCl, 50 mM NaPO₄, 250 mM imidazole) and dialyzed (4,000 MW cutoff) in buffer. The purified protein preparations were run on SDS-PAGE gels, transferred to nitrocellulose and probed with anti-His antibodies. The blot was washed and developed using chemiluminescent western blotting detection (Pierce ECL, 32106). Culture supernatants were concentrated 120-240X by precipitation with ammonium sulfate, dialyzed for 2 days in 0.1% sodium citrate, and run on 16% SDS-PAGE gels to stain secreted proteins.

Biofilm assays and microscopy. Microplate biofilms were cultivated, stained with crystal violet (CV) and quantitated as previously described (O'Toole and Kolter 1998). Briefly, 37.5 µl of CV was added to 150 µl of culture medium to a final concentration of 0.2%, and left to stain the adhered biofilm for 10 min at room temperature. The culture media and CV were removed, the wells of the microplate were rinsed 3X with distilled water (dH₂O). Plates were allowed to dry and bound CV was eluted in 175 µl of 95% ethanol. After 15 min of shaking, the blue colour in the ethanol was measured by taking OD₆₀₀ readings in the Wallac Victor³ luminescence plate reader (Perkin-Elmer). For microscopy, cultures were adhered onto glass cover slips immersed in BM2 growth

media in 6-well microplates during an overnight incubation at 37°C. Coverslips were removed, rinsed and stained with FM1-43 (5 µM), a lipophilic dye that stains the outer membrane (Lewenza et al. 2006). Biofilm images were captured with fluorescence microscopy using a Leica DMIREB2 inverted microscope and green fluorescence filters (Ex 490/20, Em 525/36), using the Quorum Angstrom Optigrid (MetaMorph) acquisition software. Images were obtained with a 63 x 1.4 objective.

Phospholipase Assay. Phospholipase C activity against the synthetic substrate p-nitrophenylphosphorylcholine (NPPC) was detected in cell-free culture supernatants as previously described (Berka et al. 1981). Briefly, one ml of overnight culture was centrifuged. The supernatant was treated with 10 mg of activated carbon to remove the culture pigment (decolorize) and re-centrifuged. Ten µl of supernatant was incubated with 90 µl of the reaction buffer containing 250mM Tris HCl, 60% glycerol, 1µM ZnCl₂ and 10 mM NPPC, for one hour at 37°C. NPPC hydrolysis was detected by measuring absorbance at 405 nm.

Aggregation and growth curves. Planktonic growth curves were performed in small volume cultures (100 µl) grown in 96-well microplates. Cultures were overlaid with mineral oil to prevent evaporation. OD₆₀₀ was monitored every 20 minutes during incubation in the Wallac Victor³ luminescence plate reader (Perkin-Elmer) at 37°C. Samples were taken from overnight microplate cultures and spotted onto 1% agarose beds on microscope slides, as previously described (Lewenza et al. 2006). Phase

contrast images were acquired using a Leica DMIREB2 inverted microscope, to visualize the state of aggregation in various culture conditions.

Statistical analysis was performed using GraphPad Prism v4.0 software. One-way ANOVA with Bonferroni post tests were performed for multiple comparisons to determine significant differences. Data were considered significant at $p < 0.05$.

Results and Discussion

Secreted phospholipases contribute to biofilm formation. We tested mutants in numerous enzymes that are secreted by the T2SS to screen for defects in biofilm formation. The panel included mutations in proteases, lipases, phosphatase, phospholipases, deoxyribonuclease, exotoxin A and a chitin-binding protein (Table 1). After growth in defined BM2 medium with high phosphate levels, most mutants had no biofilm defect, while mutants defective in the *plcB* and *plcN* phospholipases had hyperbiofilm phenotypes (Fig 1A). To confirm that the T2SS was implicated in biofilm formation, an *xcpS::lux* mutant also demonstrated a hyperbiofilm phenotype (Fig 1A). Given the established role of deoxyribonucleases in biofilm formation (Seper et al. 2011), we predicted that the EddB DNase (Mulcahy et al. 2010) would contribute to biofilm formation. However, we were unable to demonstrate a hyperbiofilm phenotype for the *eddB::lux* mutant, regardless of the phosphate concentration (data not shown). This may reflect a minor role for eDNA in this 1 day biofilm formed in microplates.

Since the PlcN, PlcH and PlcB phospholipase enzymes are induced under phosphate-limiting conditions (Barker et al. 2004), we tested the influence of phosphate

concentration on biofilm formation and showed that biofilm formation was reduced under phosphate limiting conditions (Fig 1B). Taken together, biofilm formation increases when these enzymes are absent and conversely, decreases in conditions where phospholipase production is induced.

Complementation of the *plcB* mutant restores biofilm formation. In addition to the standard CV assays for staining biofilms in microplates, we wanted to visualize the hyperbiofilm phenotype. Biofilms were cultivated in BM2 with limiting phosphate to increase expression of the phospholipases. Biofilms adhered to the surface of glass coverslips were imaged using fluorescence microscopy. Biofilms were stained with the green fluorescent lipophilic dye FM1-43, which stains the outer membrane (Lewenza et al. 2006), and any membrane debris in the extracellular matrix. Consistent with the CV biofilm phenotype, mutants in the *plcB* and *plcN* phospholipases produced large, surface-attached aggregates on glass surfaces (Fig 2A). While biofilms formed after 1 day were relatively thin and limited in complex structure, we quantitated the aggregation phenotype of glass coverslip biofilms by measuring the total fluorescence per field of view. The *plcB* mutant produced the most dramatic hyperbiofilm phenotype on both glass coverslips and in microplate biofilms (Fig 2B, 2C). To complement the *plcB* mutant, the *plcB* gene was cloned with a C-terminal His-tag directly into *P. aeruginosa* under the control of an IPTG inducible promoter. Genetic complementation restored the biofilm phenotype on glass coverslips and in microplate biofilms (Fig 2). To confirm that the phospholipase activity was due to a secreted protein, we measured phospholipase in cell-free culture supernatants. We confirmed that the *plcB* and *plcN* mutants had less

phospholipase activity than the parent strain, and that when complemented with pPlcB, phospholipase activity in the culture supernatant was restored (Fig 3A). In summary, mutants that lacked the phospholipases produced biofilms with more biomass and larger aggregates.

P. aeruginosa produces four enzymes in the phospholipase C group: PlcA, PlcB, PlcH and PlcN (Vasil 2006). Of the three phospholipase C enzymes tested here (PlcB, PlcH and PlcN), and two additional lipases (LipA and LipC), only mutations in the *plcB* and *plcN* enzymes contributed to the hyperbiofilm phenotype. One possible explanation for this finding is the substrate specificity of these enzymes. PlcH has preferred activity on phosphatidylcholine (PC) and sphingomyelin (SM) (Luberto et al. 2003), which are common phospholipids in eukaryotic cells, and may explain why this enzyme is cytotoxic to host cells. PlcH causes host tissue damage, degrades lung surfactant and contributes to lung damage during infection (Luberto et al. 2003; Vasil 2006; Wargo et al. 2011). PlcB has a broader substrate range and has hydrolytic activity against SM, PC, phosphatidylserine (PS), and is uniquely active against phosphatidylethanolamine (PE) (Barker et al. 2004). As PE is a major component of bacterial membrane lipids, this may explain why PlcB can influence the accumulation of biofilm biomass and aggregation. It is not fully understood why PlcN has an effect on biofilm formation, as it also degrades eukaryotic lipids, albeit weaker than PlcH and is therefore non-hemolytic (Ostroff et al. 1990).

Aggregation phenotype in planktonic culture of the *plcB* mutant. When monitoring the growth of the *plcB::phoA* mutant, we noticed an apparent growth defect of the *plcB*

mutant in limiting Mg^{2+} (100 μM) and phosphate (200 μM) conditions (Fig 4A). The growth defect was restored in the complemented strain (Fig 4A). We hypothesized that the altered growth kinetics of the *plcB::phoA* strain was the result of an increased aggregation phenotype, which reduced the optical density measurements. To test this hypothesis, we examined the planktonic cultures for aggregates and showed that the *plcB::phoA* strain produced larger aggregates than the wild type or complemented strains, which produced mostly individual cells or very small cell clusters (Fig 4B). The *plcN::lux* mutant had similar growth kinetics to the *plcB::phoA* mutant (data not shown) but we did not examine this strain for aggregates with microscopy. In planktonic cultures, secreted phospholipases may play a role in cell-cell detachment, suggesting a surface-localization pattern. There are examples of type II secreted enzymes attached to the outer surface, in addition to being secreted (Rondelet and Condemine 2013). The *plcB* mutant phenotype included robust biofilms formed on plastic and glass surfaces, as well as pronounced aggregates in planktonic cultures.

Exogenous phospholipase reduced the biofilm and aggregation phenotypes. We wanted to test the possibility that exogenously added phospholipase could reduce biofilm formation and aggregation, and therefore be considered as a novel enzymatic approach to dissolving biofilms. We cloned PlcB as a C-terminal His⁶-tagged protein that we tried to purify using cobalt affinity chromatography. Although the PlcB-His⁶ protein had phospholipase activity and functionally complemented the *plcB* mutant phenotypes (Figs 2, 3A), we were unable to detect the His-tagged enzyme with anti-His antibodies (data not shown). We examined the concentrated culture supernatants of the

complemented strain and were able to detect increased production of a protein band in the approximate size of the PlcB (37 kDa) (Fig 3B). While the SDS-PAGE gels indicated the increased production of an appropriate sized protein, this protein could not be purified with affinity chromatography. We therefore concluded that this His tag may have been proteolytically cleaved, leaving a functional PlcB protein intact.

To overcome this limitation, we tested the ability of commercially available phospholipase D from *Streptomyces chromofuscus* to disrupt biofilm formation. Phospholipase D was selected rather than phospholipase C, because this enzyme was available in large quantities and it has a broad substrate specificity for hydrolyzing phospholipids PC, PE, PS and phosphatidylglycerol (PG), with the highest efficiency for PC (Martin et al. 2000). Exogenous phospholipase D was able to reduce the hyperbiofilm phenotype of the *plcB* mutant, with a more pronounced effect with increasing concentrations tested (Fig 5A). We also demonstrated the ability of exogenous phospholipase D to restore normal growth kinetics (Fig 5B), which was likely due to dissolving aggregates formed of the *plcB::phoA* strain in planktonic conditions (Fig 4B).

CONCLUSIONS

Bacterial phospholipases are known for their virulence roles in invasion, damaging host cells and tissues, and nutrient scavenging (phosphate, carbon, nitrogen) (Vasil 2006). Numerous proteins and enzymes are detected in biofilms (Flemming and Wingender 2010) and here we report a novel role for phospholipases in biofilm formation. Mutation in the *plcB* and *plcN* phospholipases resulted in hyperbiofilm and

aggregative phenotypes, which suggests that phospholipases are limiting aggregate formation and degrading membrane lipids in the biofilm matrix. The bacterial phospholipid content of the biofilm matrix is likely in the form of OMVs (Schooling and Beveridge 2006; Gunn et al. 2016) or from cell membrane debris as a result of lytic cell death. Lysis is common in biofilms, resulting from autolysis, explosive lysis, DNA and phage-mediated killing, or antibiotic treatment (Allesen-Holm et al. 2006; Mulcahy et al. 2008; Turnbull et al. 2016). We propose that phospholipases with specificity for bacterial phospholipids may be employed as a novel enzymatic approach for disrupting and blocking biofilm formation.

ACKNOWLEDGEMENTS. The authors thank Steve Shideler, Mike Wilton and Tao Dong's lab for technical support with protein purification. This research was supported by an NSERC Discovery Grant. EVTB was supported by the Beverly Phillips Rising Star and Cystic Fibrosis Canada Studentships.

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FIGURE LEGENDS

Figure 1. Hyperbiofilm phenotype of mutants defective in phospholipases. Crystal violet (CV) staining of biofilms (BM2 + 1.6 mM phosphate) adhered to 96-well microplates. A. Biofilm phenotypes of transposon mutants within type II secreted enzymes. CV reads were normalized to PAO1 control and % biofilm formation values shown are the mean and standard deviation from 8 replicates (n=3). Significant differences in biofilm formation versus PAO1 are indicated: ***($p < 0.001$) and ****($p < 0.0001$). B. Effect of phosphate concentration of PAO1 biofilm formation. Values shown are the mean and standard deviation from 6 replicates (n=3). Significant difference by unpaired t-test in biofilm formation is indicated: ****($p < 0.0001$).

Figure 2. Complementation of the *plcB* mutant restores normal biofilm formation.

A. Biofilms formed on glass coverslips and imaged by staining cells with the outer membrane binding dye FM 1-43. Biofilms were cultivated overnight in BM2 succinate media containing 100 μM Mg^{2+} and 200 μM phosphate. Media was supplemented with 0.1 mM IPTG to induce plasmid expression of pPlcB. Six representative images are shown for each strain (n=3). Images were taken with the 63X objective. B. Total biofilm fluorescence (FM 1-43) as an indicator of aggregation on glass coverslip biofilms. Values shown are the mean and standard deviation from 10 fields of view per strain. Significant differences in fluorescence versus PAO1 are indicated: ***($p < 0.001$) and ****($p < 0.0001$). ##($p < 0.01$) for PlcB complemented strain versus the mutant. C. Complementation of the hyperbiofilm phenotype in microplates stained with crystal violet (CV). Values shown are the mean and standard deviation from 11 replicates

(n=3). Significant differences in biofilm formation versus PAO1 are indicated: **($p<0.01$) and ****($p<0.0001$).

Figure 3. Secreted PlcB-His⁶ has phospholipase activity. A. Cell-free supernatants were isolated from cultures grown in BM2 succinate containing 100 μM Mg^{2+} and 200 μM phosphate and assayed for phospholipase activity against the synthetic substrate NPPC. Media was supplemented with 0.1 mM IPTG to induce plasmid expression in *plcB::phoA/pPlcB*. Values shown are the mean and standard deviation from 3 replicates (n=3). Significant differences in enzyme activity versus PAO1 are indicated: **($p<0.01$). B. Concentrated cell-free supernatants were analyzed on 16% SDS-PAGE gels from the complemented *plcB::phoA* mutant (lane 1) and the mutant alone (lane 2). Sizes of relevant protein markers are indicated. The asterisk highlights the protein band (~37 kDa) that is more abundant in the supernatant of the strain expressing the PlcB-His⁶.

Figure 4. Growth and aggregation properties of the *plcB* mutant. A. Growth curves of the *plcB::phoA* mutant and complemented *plcB::phoA/pPlcB* in BM2 succinate containing 100 μM Mg^{2+} and 200 μM phosphate. Media was supplemented with 0.1 mM IPTG to induce plasmid expression in *plcB::phoA/pPlcB*. Values shown are the mean and standard deviation from 3 replicates (n=3). B. Samples were removed from the final time points of the growth experiments above and microscopically examined for aggregate formation. Representative images are shown from 6 replicates (n=3). Images were taken with the 63X objective.

Figure 5. Exogenous phospholipase D reduces biofilm formation and

aggregation. Commercial phospholipase D (*S. chromofuscus*) was added in increasing amounts to the *plcB::phoA* mutant and the crystal violet (CV) biofilms were determined. CV values (OD₆₀₀) shown are the mean and standard deviation of 10 replicates (n=3). Significant differences in biofilm formation versus PAO1 are indicated: ***($p < 0.001$) and ****($p < 0.0001$). B. Effect of exogenous phospholipase D on the growth kinetics of the *plcB::phoA* mutant. Values shown are the mean and standard deviation of triplicates (n=3).

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Figure 1. Hyperbiofilm phenotype of mutants defective in phospholipases.

183x66mm (300 x 300 DPI)

Figure 2. Complementation of the *plcB* mutant restores normal biofilm formation.

171x125mm (300 x 300 DPI)

Figure 3. Secreted PlcB-His6 has phospholipase activity.

141x61mm (300 x 300 DPI)

Figure 4. Growth and aggregation properties of the *plcB* mutant.

109x113mm (300 x 300 DPI)

Figure 5. Exogenous phospholipase D reduces biofilm formation and aggregation.

157x55mm (300 x 300 DPI)

Table 1. Strains, plasmids and primers used in this study.

Strain Name	PA ORF	Genotype	Description	Reference
PAO1			Wild type	
PW2552	PA0852	<i>cbpD::lacZ</i>	Tn mutant in chitin-binding protein, CbpD	(Held et al. 2012)
PW4283	PA1871	<i>lasA::phoA</i>	Tn mutant in protease, LasA	(Held et al. 2012)
PW7302	PA3724	<i>lasB::phoA</i>	Tn mutant in elastase, LasB	(Held et al. 2012)
PW5803	PA2862	<i>lipA::lacZ</i>	Tn mutant in lactonizing lipase, LipA	(Held et al. 2012)
PW9095	PA4813	<i>lipC::lacZ</i>	Tn mutant in lipase, LipC	(Held et al. 2012)
PW6536	PA3296	<i>phoA::lacZ</i>	Tn mutant in alkaline phosphatase, PhoA	(Held et al. 2012)
PW1025	PA0026	<i>plcB::phoA</i>	Tn mutant in phospholipase C, PlcB	(Held et al. 2012)
PW2537	PA0844	<i>plcH::lacZ</i>	Tn mutant in phospholipase C, PlcH	(Held et al. 2012)
PW3079	PA1148	<i>toxA::phoA</i>	Tn mutant in exotoxin A, ToxA	(Held et al. 2012)
19_B2	PA3319	<i>plcN::lux</i>	Tn mutant in phospholipase N, PlcN	(Lewenza et al. 2005)
76_F8	PA3102	<i>xcpS::lux</i>	Tn mutant in type II secretion system	(Lewenza et al. 2005)
41_A5	PA3909	<i>eddB::lux</i>	Tn mutant in deoxyribonuclease, EddB	(Lewenza et al. 2005)
Plasmids				
pPSV37	IPTG inducible expression vector, Gm ^R			(Lee et al. 2010)
pPlcB	pPSV37 with <i>EcoRI-HindIII</i> fragment encoding PlcB-His6x tag			This study
Primers				
F-PlcB	5'-ATGCGAATTCAGGAGGAAACGATGAAAACCTTCGCCCGCCTG-3'			This study
R-6xHIS-PlcB	5'ATGCAAGCTTTTCAGTGGTGATGGTGATGATGGAGAGCAGTCGGTGCA TCGAAG			This study