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Confirmation of Caspase-3-Like-Protease, Clp, in *Pseudomonas aeruginosa* as an Individually Regulated Gene and its Involvement in Healthy Colony Formation

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Pseudomonas aeruginosa **is an important nosocomial opportunistic human pathogen. In other studies the caspase-3-like protein Clp was determined to be required for structured biofilm formation and nutrient limited growth. In this study, the *clp : : lux* mutant phenotype previously observed was investigated by complementation analysis. Using bioinformatics we were able to identify a putative operon in which *clp* resides. A complementation analysis was performed to establish whether a *clp* mutation or a polar mutation caused the observed mutant phenotype. Colony morphology was observed by a Congo red assay. The *clp : : lux* mutant phenotype was characterized by colonies with numerous voids, frayed edges, reduced thickness and the absence of green pigmentation. The complemented mutant displayed restored wild type phenotype, flat colonies with spreading borders and green pigmentation. These observations verified that the complementation rescued the *clp : : lux* mutant, restoring the wild type Clp phenotype. Therefore, we concluded that the mutant phenotype was due to disruption of the *clp* gene, and not a polar mutation.**

Pseudomonas aeruginosa is a very common opportunistic bacterial pathogen and is the cause of many nosocomial infections (8). *P. aeruginosa* is a gram negative, aerobic, rod shaped bacterium. The colony morphology characteristics of *P. aeruginosa* include: rapidly growing, flat colonies, with a spreading border, that are often opalescent; β-hemolytic; and green pigmented (9). *P. aeruginosa* secretes a variety of pigments such as fluorescein and pyocyanin, which are important virulence factors (9). Pyocyanin impairs ciliary function of mammalian host epithelia and mediates tissue damage through production of toxic oxygen radicals (9). Pyocyanin is regulated by quorum sensing, a process involving a cell density dependent accumulation of low molecular weight signaling molecules that enable bacteria to modulate the expression of virulence genes (4). Quorum sensing is a very complicated pathway and many of its components are not fully understood. Proteases such as the caspase-3-like protein are often involved in signal cascades, such as those hypothesized to be involved in quorum sensing (4).

In previous studies researchers identified caspase-3-like protein in *P. aeruginosa* correlating to the PA4576 ORF (1). The PA4576 ORF was found to have high homology to *lon* in *P. aureginosa* and so named Caspase-3-like Lon Protease, Clp. Clp is predicted to be a ATP-dependent serine protease belonging to the Lon family and has a hypothetical serine active site. As an ATP-dependent serine protease Clp would most likely have a role in post-translational modification of proteins, protein turnover, and chaperoning. The Lon protease, which has significant homology to Clp, in *P. aureginosa* is involved in many cellular processes, including rapid cell death and quorum sensing (1). Overall the role of serine proteases can vary and a single serine protease is often not limited to a single function but is diverse in its responsibilities. It was determined in previous studies that the *clp* gene was required for normal growth in the presence of limited nutrients and the formation of structured biofilms. These researchers also observed that a *clp : : lux* mutant (c*lp*Δ517-817), produced colonies with numerous voids, frayed edges, and reduced thickness as observed by light field images (1). These morphological differences along with the high homology to Lon indicate that Clp may be involved in many cellular pathways, including rapid cell death, antibiotic resistance, and quorum sensing (1).

The *clp : : lux* mutant was generated by transposon mutagenesis, a process which often leads to polar mutations (5). A polar mutation is an upstream mutation that affects the expression of downstream genes or operons (5). These mutations tend to occur early within the sequence of genes and can be nonsense, frameshift or insertion mutations (5). Based on this knowledge we used bioinformatics techniques to investigate whether it was a *clp* mutation or an entire operon being inactivated that caused the altered phenotype observed in the *clp : : lux* mutant.

In order to confirm that the phenotype observed by Castaneda *et al*. (1) was a result of the *clp* gene mutation and not a polar mutation affecting the entire operon, we performed a complementation analysis. A complementation analysis should restore the *clp* gene, and therefore the wild type phenotype if the findings of the previous study are not due to a polar mutation. In this investigation we transformed a plasmid containing the entire PA4576 ORF into *P. aeruginosa*, via electroporation. If *clp* was the only gene affected by the transposon mutagenesis, the *clp : : lux* mutant should be rescued to wild type phenotype upon complementation with cloned *clp* gene. If the mutant phenotype was generated by a polar mutation, the complementation analysis would not be able to restore the mutant to wild type phenotype. The results indicated that the *clp* gene was involved in the process or pathway involved in the growth of structured biofilms.

MATERIALS AND METHODS

Strains and media. *Pseudomonas aeruginosa* PAO1 and mutant strain PAO1_lux_81_G7 were obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology, University of British Columbia. All overnight cultures were grown for 16 hours at 37˚C with aeration. All cultures were grown in Luria-Burtani broth (LB), with the addition of antibiotics as necessary for the individual strains (50 µg/mL tetracycline for the *clp : : lux* mutant and 50µg/mL tetracycline with 350 µg/mL carbenicillin for the *clp : : lux/*pUC19 *: : clp*$^+$) unless otherwise indicated. LB contained tryptone (1%, BD Science), yeast extract (0.5%, BD Science), and NaCl (1%). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich.

Bioinformatics analysis. The PA4576 ORF was run in NCBI BLAST program to search for known proteins with high homology. The putative operon PA4577-fklB gene sequence found in the Pseudomonas Genome Database V2 was searched using BPROM on www.softberry.com for promoter and manually searched for stop codons. The probability (p) of two or more of the genes being in an operon was analyzed using the G-Browse function on the Pseudomonas Genome Database V2.

Extraction of genomic DNA from *P. aeruginosa PAO1*. DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Catalogue No. 69504). After extraction the DNA was checked for purity through A_{260} and A_{280} readings and A_{260}/A_{280} ratio. Primer design. The following primers were designed to amplify the entire PA4576 reading frame, with the addition of cut sites for restriction enzymes XbaI on the forward primer and KpnI on the reverse primer to facilitate ligation of the amplicon into pUC19 (New England Biolabs. Cat. No. N3041L) plasmid vector. 5' primer (SHLF08-F): 5'-TTTTCTAGAATGCCCGATT CCGTCGCTGC. 3' primer (SHLF08-R): 5'-TTTGGTACCTCAGTCCTTC TTCGCCGCGG

PCR amplification. The PA4576 ORF of *P. aeruginosa* PA01 was amplified using Invitrogen Platinum® *Taq* polymerase (Cat. No. 10966-18) with SHLF08-F and SHLF08-R at a final concentration of 0.2 µM and 0.4 µM. The PCR reaction mixture included the optional enhancer provided with the enzyme. The samples were amplified for ten cycles using a touchdown annealing temp of 67-57˚C for 45 seconds and an extension temperature of 72˚C for three minutes, followed by 25 cycles at an annealing temperature of 62˚C for 45 seconds and an extension temperature of 72˚C for three minutes. After amplification the PCR products were run on a 0.7% agarose gel in 1X TAE buffer at 120V for an hour and then stained in 0.2 µg/ml ethidium bromide solution for 40 minutes to check the quality of the product.

Digestion and ligation. Both the PA4576 amplicon and pUC19 plasmid were digested for three hours at 37˚C with XbaI (Invitrogen Cat. No. 15226-012) at 1 unit/10 µl and KpnI (Invitrogen Cat. No. 15232-010) at 0.5 units/10 µL in restriction enzyme REACT 4 Buffer (Invitrogen Cat. No. 15461-023) at a total volume of 60 µL and 40 µL, respectively. Following digestion the plasmid and amplicon were cleaned using DNA Extraction Kit (Fermentas Cat. No K0513) and quantified by taking an A_{260} reading. 25 µg of digested amplicon DNA and 20 ng of digested plasmid DNA were combined in a ligation reaction using T4 ligase (Fermentas Cat. No. EL0015) as per manufacturer's protocol. This reaction was incubated overnight at 17˚C.

Electroporation was performed as described by Choi *et al.* (2). 6 mL of an overnight culture of the *P. aeruginosa clp : : lux* mutant strain was distributed equally into 4 microcentrifuge tubes and harvested at room temperature for 1-2 min at 16,000 ×g. The pellets were washed and resuspended in 1 mL 300 mM sucrose, combined, pelleted, and resuspended in 100 µL 300 mM sucrose. 50 ng of the purified plasmid was combined with 100 µL of electrocompetent cells in a 2 mm gap width electroporation cuvette. Cells were electroporated using the Biorad MicroPulser™ electroporation apparatus using the Ec2 Micropulser setting. 1 mL of room temperature LB was immediately added after the pulse and the cells were transferred to a small glass tube and incubated for 2 hours at 37ºC. At two hours, serial dilutions of the cells were plated on LB with tetracycline and carbenicillin and incubated at 37 ºC to select for successful transformants, the *clp : : lux/*pUC19 *: : clp*$^+$ strain (pUC19 *: : clp*$^+$).

Congo red assay was performed as described by Castaneda *et al.* (1). LB-Broth supplemented with Congo red (40 µg/mL, Kodak) and Coomassie Brilliant Blue (15 µg/mL, Kodak) and the appropriate antibiotics as described above were used judge pellicle morphology and color. Congo red plates contained 1.5% agar. Cells were plated by spotting 5 µL of overnight bacterial culture normalized by turbidity (OD_{460}). The plates were grown at 37 ºC to assess colony morphology.Biofilm formation assay.

Biofilm assay was performed as described by Castaneda *et al.* (1). Overnight cultures of *P. aeruginosa* PAO1, *clp : : lux* mutant strain, and pUC19 *: : clp*$^+$ strain cells were diluted 1/1000 in LB media with the appropriate antibiotics for each strain, as described above, and transferred to a 96-well plate in six replicates of 100 µL each. Cultures were incubated at 37 ºC for 24 hours. At 24 hours, cultures were removed and the wells washed twice with 150 µL of phosphate buffered saline (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4). Adherent cells were incubated with 1% crystal violet solution in 95% methanol for 15 min at room temperature, followed by three washes with PBS. Pure dimethyl sulfoxide (DMSO) was added to the crystal violet stained cells to solubilize the dye for 5 min, and this solubilized dye was transferred to new wells for taking an absorbance at 595nm (A_{595}) readings (3).

RESULTS

Bioinformatic analyses of the *Pseudomonas aeruginosa* putative operon PA4577-*fklB*. Using bioinformatics we identified a putative operon, PA4577-*fklB*, in which *clp* resided. Promoter elements and stop codons were present in the intergenic sequences between each ORF in the gene cluster. The p values of any of two adjacent genes being in the same operon in the PA4577-*fklB* gene cluster were also below 0.5.

Amplification of caspase-3 like protein gene from *Pseudomonas aeruginosa*. As shown in Figure 1, we found that touchdown PCR produced DNA bands which corresponded to the expected size of *clp*,

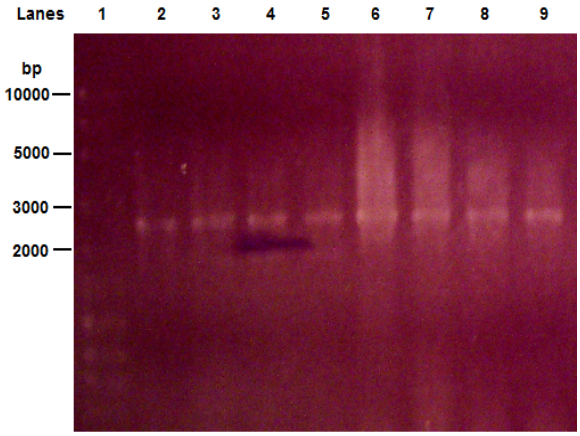

FIG. 1. PCR amplified *clp* gene under different genomic template DNA concentrations. Lane 1: MassRuler™ Express Forward DNA Ladder Mix (Fermentas, Burlington, ON, Cat. No. SM1283), Lanes 2-5: *clp* DNA amplified using 0.2 uM primer and 1/10, 1/50, 1/100, 1/500 diluted template DNA, respectively, Lanes 6-9: *clp* DNA amplified using 0.4 uM primer and 1/10, 1/50, 1/100, 1/500 diluted template DNA, respectively.

approximately 2500 bps. Increasing dilutions of genomic DNA template produced more distinctive bands for both concentration of primers used, as seen in lanes 2 to 5 and lanes 6 to 9. Lower primer concentrations increased specificity of DNA amplification, with less DNA smearing compared to higher primer concentrations.

Expression of PCR amplified caspase-3 like protein gene in *Pseudomonas aeruginosa* PAO1 *clp : : lux* mutant. Subsequent to PCR amplification of *clp*, *clp* and pUC19 plasmid was restriction digested and ligated together to produce a plasmid vector capable of expressing Clp. *P. aeruginosa clp : : lux* mutant was transformed, selected for with carbenicillin and tetracycline and isolated. The pUC19 : : clp^+ strain was then used to compare colony morphology to *clp : : lux* mutant and wild type PAO1. Wild type PAO1 showed green pigmentation, whereas there was an absence of green pigmentation in the *clp : : lux* mutant colonies and cultures. This was restored in the pUC19 : : clp^+ strain.

Comparison of colony morphology in Congo red assay. Colony morphology was further compared in a Congo red assay (Figure 2). The wild type PAO1 and the pUC19 : : clp^+ transformant were healthy with smooth edges, having circular raised structure, and no voids within the colony, while the colony morphology of the *clp : : lux* mutant were unhealthy with frayed edges, and amorphous and flat with void spots within the colony.

DISCUSSION

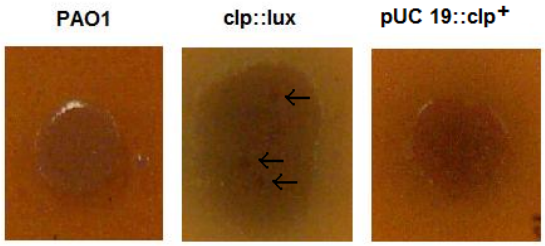

FIG. 2. The effect of *clp* complementation on the morphology of colonies in the Congo red assay. Arrows indicate void spots within a colony.

Bioinformatics analysis of the PA4576 genetic sequence associated with the *clp* gene (1) and downstream genes revealed promoters and termination sequences in positions consistent with each of these genes being individually regulated and not within an operon. The polar mutations characteristically found in transposon mutants occur when the continuity of the mutated gene is disrupted and the expression of downstream genes in the same operon is abolished by the insertion of the transposon in the upstream gene (11). Our findings strongly supported that the phenotypic changes observed in the previous study were caused by the Clp Δ517-817 mutation in the *clp : : lux* mutant and were not a result of frameshift mutations in downstream genes.

The colony morphology of the PA01 and pUC19 : : clp^+ strains was similar, while the *clp : : lux* mutant colony morphology was notably different. Specifically, a lack of green pigmentation was noted in the colonies of the *clp : : lux* mutant. This pigment is probably pyocyanin (8). Although the genes involved in the biosynthesis of pyocyanin are well characterized (8), its synthesis is regulated by one of two complex and interrelated acyl-homoserine lactone quorum-sensing-signaling systems in *P.aeruginosa* (8, 10). These observations indicate that Clp may be involved in quorum sensing through the regulation of the production of this virulence factor. Since quorum sensing is also involved with biofilm formation (4), this explanation also correlates with previous findings (1), that Clp is required for motility-independent structured biofilm formation.

It is known that *P. aeruginosa* colonizers often convert from low producers of the capsular polysaccharide alignate to high producers of alignate, resulting in mucoid colony morphology. It should be noted that only wild-type and the complemented PA01 strains formed mucoid colonies and showed signs of increased production of exopolysaccharide alignate. In the mutant strain the mucoid phenotype was absent. During the course of lung infections in cystic fibrosis patients, *P.* areuginosa often changes from being a low to a high producer of the exopolysaccharide alignate,

and this change is often synonymous with clinical deterioration of the patient (13) The overproduction of alignate is thought to facilitate the formation of *in vivo* biofilms that helps to protect bacteria from host defenses and antibiotic treatement (13). Therefore Clp may be playing a role in regulating mucoid conversion, which may explain decreased thickness and structure observed in the Clp mutant strain by Castenda *et al*. in previous studies.

The biomass assay preformed by Castaneda *et al.*, which showed significant decrease in adherent biomass in the *clp : : lux* mutant compared to wild type PAO1, was repeated during this investigation and showed inconclusive results (data not shown). The biomass assay was performed in the presence of selective antibiotics for each respective strain unlike the previously performed assay. The presence of antibiotics can affect the growth and metabolism of bacteria (12). Given that the pUC 19 : : clp^+ strain was grown in 50 µg/mL tetracycline and 350 µg/mL carbenicillin, *clp : : lux* mutant was grown in 50 µg/mL tetracycline, and the wild type PAO1 was not grown in antibiotics, these differences in the growth media may have resulted in the individual strains are not quantitatively comparable to each other within this experiment, nor those found in the previous study. Also, all absorbance readings for this assay were above 2.0 and therefore the accuracy of the results was questionable.

The pUC 19 : : clp^+ strain grew on selective LB agar containing selective carbenicillin indicating that the mutant contained the pUC19 plasmid. The restored wild type PAO1 phenotype observed in the Congo Red assay also indicated that the pUC 19 : : clp^+ mutant contained the vector with the ligated PA4576 insert. Although these observations suggest that our transformation was successful, we were unable to recover the plasmid from the transformants used in this analysis (data not shown). Usually, plasmid extractions are done on bacterial cell cultures grown up to 16 hours. The cultures that were used for the plasmid extraction were 30 hours old and therefore may not have been suitable for plasmid extraction.

The first subculture of the pUC19 : : clp^+ colonies was done within 24 hours and grew successfully. All subsequent subcultures preformed from pUC19 : : clp^+ colonies or liquid culture grown for over 24 hours were not viable. This observation could be explained by the high copy number of replicative plasmid used in the transformation, which may have resulted in overexpression of Clp. As Clp is a putative protease and is hypothesized to be involved in multiple cellular pathways, its overexpression may have killed the cells once its levels reached a certain threshold. Upon cell death, plasmids may be degraded by DNAses in the cell and surrounding environment (6). All of the above could explain the absence of growth in the subculture of transformant cells from cultures or colonies incubated for over 24 hours, as well as our inability to recover plasmid DNA.

We conclude that the mutant phenotype observed by Castaneda *et al.* was due to disruption of the *clp* gene, and not a polar mutation and that the *clp* gene may potentially have a role in a quorum-sensing pathway regulating the expression of pyocyanin and mucoid conversion.

FUTURE EXPERIMENTS

The purpose of this experiment was to confirm that the *clp* gene alone was responsible for the *clp : : lux* mutant phenotype and not a polar mutation affecting downstream genes. We were able to perform a successful complementation assay but we were not able to confirm the presence of the wild type PA4576 gene within the *clp : : lux*/pUC19 *: : clp*$^+$ transformant. We believe that the cultures used for the plasmid extraction were too old to be viable for this purpose. Therefore this would be the first step in continuing this project. Also, we were unable to get access to a confocal microscope, in the future structured biofilm formation could be observed in this way to confirm restoration of the wild type phenotype in the complemented mutant.

In this study, we observed a lack of green pigmentation in the *clp : : lux* mutant, inferring a possible role of *clp* in the production of pyocyanin. Pyocyanin produces blue pigmentation which absorbs light at 695 nm. To show that the *clp : : lux* mutant was deficient in the production of pyocyanin, absorbance readings at A_{695} could be taken of overnight cultures of wildtype PAO1 and *clp : : lux* to assess the presence of pyocyanin.

The *clp* gene has significant homology to the *lon* gene in *P. aeruginosa* and *lonB* in *Escherichia coli.* Both *lon* and *lonB* have many different cellular roles and therefore we hypothesize that *clp* also may have many distinct roles within the cell. It would be insightful to perform a microarray analysis, comparing the wild type PA01 to the c*lp : : lux* mutant, under varying conditions such as: nutrient limitation and antibiotic treatment. By observing the presence or absence of *clp* in varying conditions researchers, will be able to elucidate any relationships between *clp* and other gene expression.

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