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Characterization of the relationship between *Pseudomonas aeruginosa* Caspase-3-Like-Protease with Biofilm Formation and Quorum Sensing

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Biofilm formation by *Pseudomonas aeruginosa* has been accepted as an important determinant to its virulence and pathology in clinical settings. It has been reported that quorum sensing (QS) mediated by the *rhl* and *las* systems, along with their cognate acyl-HSL autoinducers, play a significant role in the formation of biofilm structures. Further, it has been proposed that caspase-3-like-protease in *P. aeruginosa* has an essential role in establishment of biofilm formation in the early stages. In this study, we have attempted to demonstrate the interplay between QS genes and recently-identified PA4576 gene, putatively encoding the caspase-3-like-protease. Wild type *P. aeruginosa* grown in biofilm-inhibitory M9 minimal media was found to express caspase-3-like-protease. Using the mini-Tn5-*lux*CDABE fusion PAO1_lux_22_A5 and PAO1_lux_81_G7 mutants, comparable or enhanced biofilm formation was observed in PA4576 mutants compared to wildtype. Perplexingly, biofilm formation in both PAO1 and mutants was not found to be enhanced by the addition of exogenous acyl-HSL. Taken together, these data suggest that PA4576 gene may not be responsible for expression of caspase-3-like-protease in *P. aeruginosa*, or that the protease has weak association with biofilm formation in nutrient limited growth.

The aggregation of prokaryotes leading to formation of biofilms is a protective strategy utilized by virtually all bacterial species, by providing mechanistic stability, resulting in tolerance from extreme physiological conditions (3). The durability of biofilms in *Pseudomonas aeruginosa,* a Gram-negative species associated in nosocomial infections, is known to be particularly significant, as the formation of *P. aeruginosa* biofilms has been implicated as a major cause of catheter-associated urinary-tract infections, with high mortality and morbidity (12). The same biofilms are also responsible for the colonization, antibiotic resistance and immune evasion of *P. aeruginosa* in airway mucous of cystic fibrosis patients, exacerbating their symptoms (6).

The establishment of biofilms requires communication between cells in their planktonic states, which in many Gram-negative bacteria, is dependent upon quorum sensing (QS) of the autoinducer N-acyl-L-homoserine lactone via a two-component signalling system (3). In *P. aeruginosa,* this involves two systems: the *las* system, consisting of regulatory element LasR and its exogenous upregulator N-(3-oxododecanoyl) homoserine lactone (C12-HSL), produced by autoinducer LasI, and the *rhl* system, consisting of RhlR receptor, RhlI autoinducer and N-butyryl-L-homoserine lactone (C4-HSL). This model is supported by gene expression microarray assays showing upregulation of QS-regulated genes during induction of *lasI, rhlI* deficient PAO-JP2 strains by their cognate HSL signals, and the observation that QS-deficient mutants produce biofilms with lower biomass (14, 15, 17). This effect can also be observed following treatment with LL-37, a cationic host defence peptide, which has potent inhibitory effect on both new biofilm formation and pre-existing biofilms (13). Mutations in *lasI* and *rhlI* attenuate virulence of *P. aeruginosa*, resulting in better induction of humoral antibody titres, stronger leukocyte response and milder lung pathology in animal infection models (19).

The important roles of proteases in bacterial biofilm formation have begun to emerge. Recently, the presence of Lon protease gene PA1803, a conserved class of ATP-dependent protease with primary functions in abnormal protein degradation, was described in *P. aeruginosa* (11). The putative role of this protease in biofilm formation was demonstrated in a study which reported that PA1803 gene fusion mutant show significantly reduced biofilms formation and cell motility (11). Castaneda *et al* (1) have previously identified a putative caspase-3-protease gene, PA4576, which has been speculated to be involved in early establishment of biofilm and induction of autolysis in *P. aeruginosa*. The authors observed lowered annexin V binding, decreased biomass and reduced biofilm thickness in PA4576 *lux* gene fusion mutants, though it appeared bacterial swarming, swimming and twitching were not affected (1). The authors had used the

abbreviation *clp* to describe the gene identified, which we have refrained from using due to confusion with Clp chaperone protease, an unrelated class of ATP-dependent protease in a variety of bacterial species (8).

Given the intricate relationship between QS systems and biofilm formation and survival, we attempted to investigate the relationship between the newly identified caspase-3-like-protease in *P. aeruginosa* and HSL, and specifically, to study whether the protease falls in the same pathway as the *rhl* and *las* QS autoinducers. Using PA4576mutants, we found comparable biofilm formation in PAO1 and PA4576 mutants, in disagreement with results reported by Castaneda *et al* (1); contrary to our expectations, addition of biofilm inducing HSL failed to elevate these biofilm levels. Intriguingly, we detected caspase-3-like protease from PA4576 mutant cells grown overnight in LB, suggesting incomplete knockout of the protease in these cells. Further, we have also examined PAO1 wildtype grown in non-biofilm inducing M9 media, and found caspase-3-like-protease expression. When taken together, our results suggest that PA4576 is not involved in expression of caspase-3-like-protease in *P. aeruginosa*, and that this gene is not implicated in biofilm formation and survival.

MATERIALS AND METHODS

Strains. *P. aeruginosa* PAO1 H103 (WT) and PA4576 mutant strains, PAO1_lux_22_A5 (A5) and PAO1_lux_81_G7 (G7), were provided by the Robert Hancock Laboratory (Department of Microbiology and Immunology, University of British Columbia). The A5 and G7 mutant strains originated from UBC's lux mutant library, and differed in amino acid sequence length of the caspase-3 like protease by Δ672-817 and Δ517-817 respectively (1, 9).

Growth conditions. Overnight cultures were grown at 37 °C at 200 rpm. Two different types of liquid media were used: Luria-Bertani medium (LB) and M9 media with glycine. LB media contained 1% tryptone (BD Science), 0.5% yeast extract (BD Science), and 1% NaCl. M9 media contained 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 1mM $MgSO_4 \cdot 7H_2O$, 0.1mM $CaCl_2$, 2% glucose, and trace elements solution ($FeCl_3$, $MnSO_4$, $ZnSO_4$, $CaSO_4$, H_3BO_3, Na_2MoO_4, $COCl_2$) (1).

Biofilm formation assay. N-butyryl-L-Homoserine lactone (C4-HSL) and N-3-oxo-dedecanoyl-L-Homoserine lactone (3-O-C12-HSL) from Cayman chemicals (Cat. no. 10007898 and 10007895 respectively), were provided by Robert Hancock Laboratory, and were dissolved in DMSO at 22.4 mM and stored at -20 °C. *P. aeruginosa* PAO1 or PA4576 mutant strain overnight cultures were normalized by cell optical density (OD 460) and diluted 1/500 in either LB or M9 media followed by transfer of 100 µl of the diluted samples into 96-well polystyrene plates (BD Biosciences), with or without addition of 50 µM of C4-HSL or 3-oxo-C12-HSL. Addition of the anti-biofilm peptide LL-37 (20 mg/ml) was also used to serve as negative control. Plates were parafilm sealed, and incubated for 24 hr at 37 °C. After the incubation, cells were washed stringently with ddH_2O and stained with 110 µl of 1% crystal violet (Fisher Chemical, Cat. no. C581-25) in 95% ethanol for 20 min at room temperature. Cells were then washed with ddH_2O followed by 20 min incubation with 120 µl of 95% ethanol. The solution was transferred to new polystyrene plates for absorbance reading at 595nm (1).

SDS-PAGE. Overnight cultures of *P. aeruginosa* PAO1 and PA4576 mutants were diluted 1/500 or 1/1000 in LB media. Strains grown in M9 media were not diluted. At 24 hr after initial seeding of cells into media, cultures were microcentrifuged at 12,000 x g for 10 min to collect the cells and remove the supernatant. Each cell pellet was washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and pH 7.4) and centrifuged at 12,000 x g for 10 min. Remaining cell pellet was lysed on ice for 20 min with lysis buffer (50 mM Tris-HCl [pH 7.2] containing 2% Triton X-100, 60 mM KCl, 2 mM EDTA), with occasional mechanical disruption with pipette. Cell debris were spun down via centrifugation at 12,000 x g for 10 min and supernatants were collected. Lysates were then mixed with an equal volume of 2x sample buffer (0.5 M Tris [pH 6.8], 20% SDS, 40% glycerol, 0.1% bromophenol blue and 600 mM ß-mercaptoethanol), heated at 95 °C for 10 min. After heating, samples were immediately chilled on ice for 5 min, and then centrifuged briefly. Protein concentrations were normalized using the Bradford protein assay (10). Samples were loaded into a 12% (w/v) Tris-glycine SDS-polyacrylamide gel, and ran vertically at 120 V on a polyacrylamide gel electrophoresis system.

Western blotting. Rabbit polyclonal anti-caspase-3 antibody was purchased from Santa Cruz Biotechnology (Cat. no. sc-7148). AP-conjugated goat anti Rabbit IgG H+L was purchased from Jackson ImmunoResearch Laboratories (Cat. no. 111-001-003), and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) was purchased from Thermo Scientific (Cat. no. 34042). Hut 78 lysate was purchased from Santa Cruz Biotechnology (Cat. no. sc-2208). Electroblotting was performed using a nitrocellulose membrane (Bio Rad, Cat. no. 162-0232) in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) employing either a 110V for 45 min at room temperature or 35 V at 4 ^oC, overnight. Membrane was blocked with 5% skim milk powder in Tris-buffered saline (TBS)- Tween 20 (0.1%) for 1 hr at room temperature. Blotted membranes were incubated overnight with anti-human caspase-3 antibody that had previously between shown to cross react with *P. aeruginosa* caspase-3-like protease (1). in a 1/200 dilution in TBS-T with 1% bovine serum albumin (BSA) as recommended by manufacturer. Six 10min washes in TSB-T were performed after. Alkaline-phosphatase conjugated goat anti rabbit secondary antibody was added at 1/10,000 dilution with 1% BSA for incubation at 1 hour in room temperature (1). The blot was washed six times with TBS-T for 10 min each, and detected using 5-bromo-4-chloro-3-indoyl phosphate (BCIP+NBT).

RESULTS

Detection of caspase-3-like protease in mutant strains of *Pseudomonas aeruginosa*. Using Western blot, the expression of caspase-3-like protease in PAO1 following overnight growth in LB in two strains with mutations in the putative caspase-3-like protease gene—namely the PA4576 mutants—was examined. The two strains of PA4576 mutants were PAO1_lux_22_A5, or A5 mutant strain, and PAO1_lux_81_G7, or G7 mutant strain. Since the primary antibody used in this experiment was the anti-human caspase-3 antibody, which cross reacts with bacterial caspase-3-like protease, the positive control used was the Hut 78 lysate. Hut 78 is a human T cell lymphoma cell line that expresses caspase-3 and caspase-3-like protease. Fig. 1 shows the lane with the positive control. The 85 kDa and 60 kDa bands both correspond to the caspase-3-like protease while the 30 kDa band corresponds to the caspase-3 protein. On the other hand, the two PA4576 mutants grown in LB showed two molecular weight bands of 85 and 60 kDa,

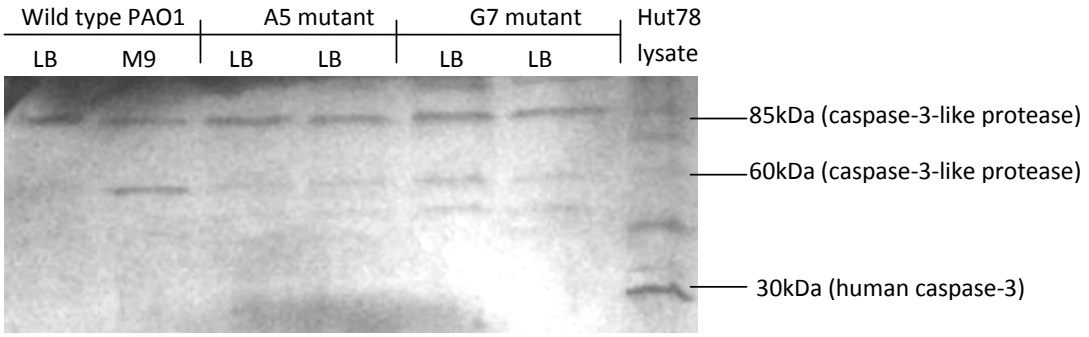

FIG. 1. Western blots showing the expression of caspase-3-like protease in *Pseudomonas aeruginosa*. The indicated POA1 strains were grown in either LB or M9 media.

which represented the bacterial caspase-3-like protease (Fig. 1). The 85 kDa band was relatively higher in intensity compared to the 60 kDa band. Wild-type PAO1 grown in LB and M9 both detected the 85 kDa band but only the M9 revealed a high intensity 60 kDa band relative to the 60kDa bands in the mutants. The 60kDa band under the wild-type culture grown in M9 was unexpected. The PA4576 mutants were not grown in M9 media to test the expression of caspase-3-like protease because the assumption was made that M9 media would not induce the expression of caspase-3-like protease in the mutants.

Effect of caspase-3-like protease and acyl-HSLs on biofilm formation in *Pseudomonas aeruginosa*. The biofilm formation assay was employed to measure the relative biofilm formation in wild-type PAO1 and the two PA4576 mutants, A5 and G7, with and without treatment of acyl-HSLs. As a control to confirm that the biofilm formation assay was able to detect biofilm reduction, the wild-type culture was treated with LL-37, a human host defence peptide that is able to interfere with the formation of *P. aeruginosa* biofilms by decreasing attachment and influencing quorum sensing systems (13). This control had to be done in a separate polypropylene plate due to the cationic nature of LL-37 which causes binding to polystyrene plastic. As expected, a reduction in biofilm formation in the LL-37-treated wild-type POA1 culture was detected in Fig. 2A, demonstrating a 43% biofilm inhibition. Contrary to the previously described result of PA4576 mutants being defective in biofilm formation (1), a trend of higher biofilm formation in the PA4576 mutants compared to the wild-type PAO1 strain was observed, showing an approximately 18% increase in biofilm formation (Fig. 2A). In Fig. 2B, the wild-type and mutant cultures were treated with different types of homoserine lactone—C4 or C12—or a combination of homoserine lactones—C4 and C12—to investigate whether this can restore or enhance biofilm formation in the putative caspase-3-like protease gene mutant *P. aeruginosa*. For the most part, the results showed that the addition of HSL to wild-type and PA4576 mutants did not improve the cultures' ability to form biofilms. However, the addition of C12 did induce a modest increase of 40% in biofilm formation. Furthermore, there was a general trend that G7 mutants produced more biofilm compared to wild-type POA1 and A5 mutant strain. Since the acyl-HSLs were dissolved in DMSO, treatment of wild-type and mutant cultures with DMSO alone was tested as a control (data not shown), but the contribution of DMSO did not produce any significant changes compared to wild-type.

DISCUSSION

The presence of a caspase-3-like protease had been previously described in wild type *Pseudomonas aeruginosa* strain PAO1 lysate as 60 kDa and 85 kDa bands detected by anti-human capase-3 antibody (1). In this study, we have also observed the 60 kDa and 85 kDa caspase-3-like protease bands in the lysates of *P. aeruginosa* (Fig. 1). It has not been determined which of the two bands actually represents the previously described caspase-3-like protease or whether they are two different forms of the same protein. Bacterial caspase was reported in *Xanthomonas campestris*; however, this protein appeared to be better defined as a single protein band at 55 kDa (5). Castaneda *et al* predicted that *P. aeruginosa* caspsae-3-like protease was encoded by the PA4576 gene, which encoded for a probable ATP-dependent protease and has homology to the *lon* gene, which also encoded for ATP-dependent proteases (1). We further characterized the expression of caspase-3-like protease in transposon insertion mutants of PA4576 gene, which had not been characterized previously. Surprisingly, western blots of the lysates of these mutants demonstrated the same two cross-reactive bands to anti-human caspase-3 antibody (Fig. 1). This led to two possibilities: either the shortened partial product of PA4576 in the mutants

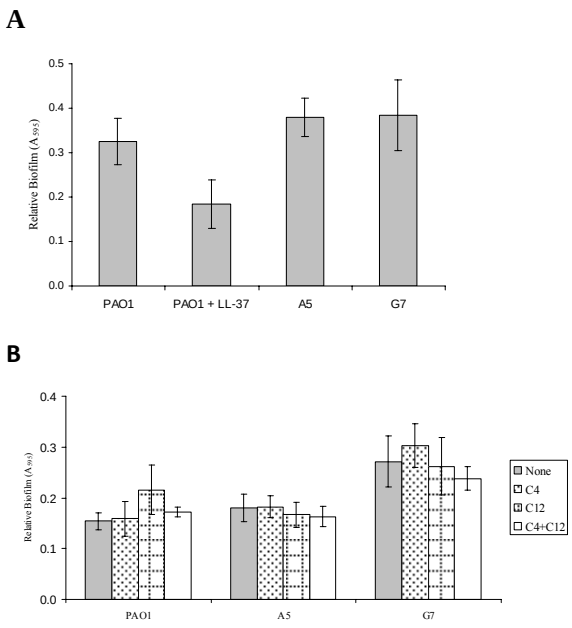

FIG. 2. Effect of PA4576 gene mutations on biofilm formation by *P. aeruginosa*. Biofilm assays were carried out using overnight cultures of indicated strains. Cells were treated with LL-37 (20 mg/ml), or an acyl-HSL (50μM in 0.2% DMSO), as indicated in the figure legend. Plotted data shows the means ±SD of five replicate wells.

were expressed and were not shortened enough to appear as caspase-3-like protease bands with lower molecular weights , or that caspase-3-like protease was not encoded by the gene PA4576. The partial products encoded by the two PA4576 mutants have 145 and 300 amino acids out of 817 amino acids left out from the predicted protein sequence encoded by the PA4576 gene (1). The mutant proteins of PA4576 would visibly have lower molecular weights than the wild type on a Western blot. Our result therefore suggested that the protein bands detected by anti-human caspase-3 antibody were not encoded by PA4576. Caspase-3-like protease was thus unlikely to be encoded by PA4576.

Castaneda *et al.* (1) suggested that caspase-3-like protease was required for the formation of *P. aeruginosa* biofilms for their possible role in rapid cell death (RCD), a programmed death of bacterial cells necessary for biofilm progression and survival. Here, we showed the expression of caspase-3-like protease in the lysate of *P. aeruginosa* grown in M9 + glycine media, a simple minimal media that did not induce biofilm formation *P. aeruginosa* grown in M9 had no signs of biofilm, while those grown in LB visibly formed biofilms. It was interesting to note that in our experiment, the wild type *P. aeruginosa* grown in LB, a biofilm inducing media, expressed only the 85 kDa caspase-3-like protein band (Fig. 1). This is in disagreement to the previous results by Castaneda *et al* (1). The 60 kDa caspase-3-like protease band in the lysate of *P. aeruginosa* grown in M9 was surprisingly more intense (Fig. 1). This indicated that M9 media may induce the expression of caspase-3-like protease, and that the expression of caspase-3-like protease did not correlate with biofilm formation. This suggested that the possible role of the caspase-3-like protease on biofilm formation was not regulated through its expression.

The ability of PA4576 mutants to form structured biofilm had been reported to be defective (1). We have on the contrary observed that PA4576 deletion mutants did not demonstrate impaired biofilm formation. Biofilms were visible when these mutants were grown in LB. Crystal violet biofilm formation assay showed that these mutants had equivalent biofilm formation to wild type *P. aeruginosa* (Fig. 2B). In addition, the G7 PA4576 mutant showed a trend of superior biofilm formation compared to wild type (Fig. 2). This was in disagreement with the previously reported results by Castaneda *et al* (1). This could be due to differences in experimental methods as our biofilm formation assay protocol called for more stringent and thorough washes. We also normalized our biofilm formation assay by adding equivalent amount of cells to each well. Our data suggested that the previously suggested importance for the PA4576 gene in the formation of structured biofilms should be reassessed. In agreement to our data, PA4576 had not been reported as one of the many genes that are differentially expressed in *P. aeruginosa* biofilms, indicating that this gene would be unlikely to be involved in biofilm formation (17, 18). The role of caspase-3-like protease on biofilm formation thus had to be reassessed as well, especially if PA4576 in fact did not encode for the protease.

Quorum sensing (QS) mediated by HSL was known to be necessary for biofilm formation in *P. aeruginosa* (2). The main acyl-HSL QS systems in *P. aeruginosa* are Las and Rhl QS systems. Las involves C12-HSL, while the Rhl system involves C4-HSL (4). Here, we have attempted to dissect the possible role of acyl-HSL mediated QS in the involvement of caspase-3-like protease in the formation of *P. aeruginosa* biofilms. We had found exogenous HSL could not enhance the biofilm formation of PA4576 mutants (Fig. 2B). The addition of HSL to wild type *P. aeruginosa* had also failed to enhance its biofilm formation. Our results suggested that mutants of PA4576 were similar to wild type *P. aeruginosa* that they already have intact and functional biofilm regulation by the HSL QS systems and did not need the addition of exogenous HSL in order to effectively form biofilms. Furthermore, PA4576 expression has not been reported as a QS regulated gene (7). If PA4576 gene is not regulated by QS, then there was no reason for HSL treated wild type and PA4576 mutants to behave differently in this

biofilm formation assay. This did not provide us any clues to how caspase-3-like protease may play a role in biofilm formation as the loss of intact PA4576 product may not translate to caspase-3-like protease deficiency.

We sought to investigate and dissect the possible relationship between caspase-3-like protease, biofilm formation and HSL mediated QS in *P. aeruginosa*. We had instead acquired four findings contradictory to the previously published study by Castaneda *et al* (1) that further convolutes the knowledge surrounding *P. aeruginosa* caspase-3-like protease. First, we discovered that *P. aeruginosa* caspase-3-like protease was unlikely to be encoded by PA4576. Mutants of PA4576 then may be the wrong model to use for studying caspase-3-like protease. An explanation to the two protein bands that cross-react to anti-human caspase-3 antibody is still needed to further study caspase-3-like protease. Since there are no antibodies specific to *P. aeruginosa* caspase-3-like protease, specificity becomes an issue; hence, it is also unclear whether these bands represent proteins detected by anti-human caspase-3 antibody or by the secondary anti-rabbit IgG antibody at this point. A simple test by using only secondary antibody alone would distinguish between the two. These could represent two different proteins, or a single protein in different states. It is unclear which of these bands defines active *P. aeruginosa* caspase-3-like protease. Second, we had found that the expression of caspase-3-like protease did not correlate with biofilm formation; hence, the previously suggested potential role of this protease in biofilm formation is not regulated through its expression. Third, the previously described defect of PA4576 mutants in the formation of biofilm may not hold true. Fourth, we had found that PA4576 deletion mutants have intact and functional biofilm regulation by the HSL QS systems and did not need exogenous HSL to effectively form biofilms. These series of findings all together suggests that caspase-3-like protease in *P. aeruginosa* is not nearly as well defined as *X. campestris* caspase (5) and much work needs to be done to clarify and define the identity and functions of *P. aeruginosa* caspase-3-like protease. Whether a relationship between caspase-3-like protease, biofilm formation and quorum sensing exists or not remains to be answered. Proper identification of caspase-3-like protease and its mutants may be necessary to answer this question.

Our findings have proven that the identity and functions of caspase-3-like protease in *P. aeruginosa* has not yet been sufficiently defined, and its suggested role in biofilm formation may need to be reassessed. Our goal was originally to study the possible relationship between the previously suggested role of caspase-3-like protease on biofilm formation, and the role of HSL mediated quorum sensing in biofilm formation of *P. aeruginosa*. We conclude that *P. aeruginosa* caspase-3-like protease would have to be further defined and characterized first before we can properly study the relationship between caspase-3-like protease, biofilm formation and quorum sensing.

FUTURE DIRECTIONS

In the effort to investigate the interplay between caspase-3-like protease with biofilm formation and acyl-HSL quorum sensing systems, a true caspase-3-like protease mutant should be identified. This could be done by first using the anti-human caspase-3 antibody to immunoprecipitate caspase-3-like protease and isolate this protein, which could then be sequenced. This sequence could be used to predict the gene encoding caspase-3-like protease in *P. aeruginosa* genome. A mutant for that gene can be obtained from existing mutant libraries or made by targeted transposon insertion. Western blots could be performed using anti-human caspase-3 antibody to confirm that these mutants lack the full length caspase-3-like protease. Once a proper mutant is obtained, commercially available caspase-3 activation assay kits could be attempted to investigate the caspase-3-like activity on these mutants compared to wild type *P. aeruginosa*; however, specificity is an issue here as these kits are made for human caspase-3 activity. Furthermore, the relationship between caspase-3-like protease and biofilm formation could be properly investigated. The crystal violet biofilm formation assay could determine whether these mutants are truly defective in biofilm development by comparing the extent of biofilm formation in LB compared to wild type *P. aeruginosa*.

Once the basic understanding of caspase-3-like protease expression has been attained, and its involvement in biofilm formation re-established, the role of acyl-HSL on biofilm formation in caspase-3-like protease deficient mutants should be examined. To study the relationship between HSL mediated QS systems and the expression of caspase-3-like protease, its expression can be tested in QS deficient *P. aeruginosa* mutants via western blots with anti human caspase-3 antibody. Furthermore, exogenous HSL treatments can be performed on *P. aeruginosa* to see whether turning up the HSL QS systems has an effect on the expression of caspase-3-like protease as detected by western blots. This will provide a better insight to the role and regulation of caspase-3-like protease in *P. aeruginosa* and answer the question whether caspase-3-like protease expression is mediated by quorum sensing. Can addition of exogenous HSL enhance caspase-3-like protease deficient mutants in biofilm formation? The uncertainties on the role of caspase-3-like protease in biofilm formation and its possible connection to

quorum sensing warrants further studies to be performed.

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