Journal of Experimental Microbiology and Immunology (JEMI) Vol. 11:23-29

Identification of a Novel Caspase-3-Like Protease, Clp, in *Pseudomonas aeruginosa* Biofilms and its Modulation of Rapid Cell Death

RACHELL J. CASTANEDA, SPENCER A. FREEMAN, MORGAN RIGGAN AND GEORGE X. SONG-ZHAO

Department of Microbiology & Immunology, UBC

***Pseudomonas aeruginosa,* a major cause of nosocomial infections, is able to evade the host immune response and confer antibiotic resistance by undergoing the dynamic process of biofilm formation. It has previously been shown that autolysis or rapid cell death (RCD) may be an important part of biofilm survival during conditions of nutrient limitation by releasing nutrients and forming channels for improved nutrient flow. Details of RCD in *Pseudomonas* have not been elucidated but evidence suggests that expression of caspase-like proteins and other proteases may be involved. Here, we have demonstrated the inducible expression of caspase-3-like proteins upon biofilm formation. Using the mini-Tn*5-luxCDABE* fusion PAO1 library, we have specifically identified Clp, a caspase-3-like protein with homology to the Lon protease of *Pseudomonas* and LonB in *Escherichia coli,* to be required for motility-independent structured biofilm formation. Our data suggest that Clp may be involved in a proteolytic cascade leading to RCD in nutrient limited growth of *P. aeruginosa*.**

The formation of biofilms is fundamental to the survival of a broad range of microorganisms in diverse environmental, industrial and clinical settings (5). Biofilm formation and bacterial community structure have been at the centre of intense recent study, with the hope of gaining insight leading to antibiofilm therapies. Biofilm formation in *Pseudomonas aeruginosa* leads to antibiotic resistance development in cystic fibrosis patients and enhanced bacterial survival against the host immune response (6, 20). Studies on the survival of bacterial cells in a large biofilm structure have led to the idea that autolysis or rapid cell death (RCD), comparable to programmed cell death in eukaryotes, may be a critical strategy used in biofilms to overcome limited nutrient diffusion throughout the biofilm (26, 29). By this theory, RCD within a biofilm would increase overall survival of the bacterial community at the expense of individual bacterium through the release of nutrients and DNA and the generation of channels that may allow for better nutrient flow through the biofilm structure (19). Recently, studies have identified the molecular events underlying RCD in *Xanthomonas*, an organism that infects plant tissues, and have shown that these correspond to increased expression of a caspase-3-like protein (25). Other studies have shown a caspase-3-like protein to be expressed during early stages of biofilm formation in *Pseudomonas aeruginosa*; however no caspase-like gene has been identified and the general process of RCD remains poorly understood (14).

There is an observed gradient of biofilm phenotypes for *Pseudomonas aeruginosa*, ranging from a uniform confluent film to a structured biofilm, consisting of cell aggregates or "mushrooms" separated by channels (24). Several factors have been shown to influence whether *P. aeruginosa* forms flat or structured biofilms, inclusive of the nutritional environment and gene expression (24, 10). For example, grown with glucose as the sole carbon source, *P. aeruginosa* forms structured biofilms, while glutamate- and succinate-grown biofilms are flat (11). Direct cell and whole-cell protein analysis have shown five stages in *P. aeruginosa* biofilm development: (i) reversible attachment, (ii) irreversible attachment, (iii) maturation-1, (iv) maturation-2, and (v) dispersion (28). The transcriptional events and environmental requirements mediating biofilm maturation are only recently being determined. For example, *pilA* and *rhlA* are required for microcolony formation in the early phase of *P. aeruginosa* biofilm development and normal migration-mediated structure formation in the later three stages of biofilm development (24). Specifically, PilA and FliM mediate twitching motility, the mechanism used by *P. aeruginosa* to find initial biofilm formation sites and to "climb up" the initial monolayer of cells, forming the "cap" of structured biofilms (22, 29). The expression of genes required for normal biofilm formation depends, at least in part, on the nutritional environment (22, 29). The *crc* modulon may be critical for biofilm formation through the regulation of amino acid, carbon-metabolism, and type-IV pilus genes where expression has been shown to be dependent on available carbon-sources (23). *Xanthomonas* requires protein-rich media or particular

amino acid supplemented media and high intracellular pyruvate, cyclic-adenosine monophosphate (cAMP), and citrate concentration for biofilm formation (25).

The role of proteases in biofilm formation is unclear. Studies have recently shown the Lon protease to be required for robust biofilm formation and motility in *Pseudomonas*, though this mechanism may be either active, homeostatic, or both (20). Caspase-3 is part of the aspartate-specific cysteinyl protease family in eukaryotes, where the activation of intracellular signaling mediates its involvement in apoptosis (13). Recent studies have identified caspase-3 like proteins in *Xanthomonas* (8) and *P. aeruginosa* (14). Here, we have confirmed the inducible expression of caspase-3-like proteins in *P. aeruginosa* biofilm formation and have used a mini-Tn*5*-*luxCDABE* fusion PAO1 library to find enzymes with similar active sites to caspase-3. In this search, we have identified a caspase-3-like protease with homology to the *lon* gene in *P. aeruginosa* and high homology to *lonB* in *Escherichia coli*, which we refer to as Clp (Caspase-3-like Lon protease). Using *clp* : : *lux* mutants, we have characterized this protease as being required for nutrient limited growth and structured biofilm formation in a motility-independent mechanism. Moreover, we have shown Clp to significantly mediate caspase activity during early biofilm establishment, correlating with apoptotic markers. Taken together, our data suggest there may be a cascade of proteolytic activity involving Clp in *P. aeruginosa* which is required for structured biofilm formation, and may be a potential target for future anti-biofilm therapies.

MATERIALS AND METHODS

Strains and chemicals. *P. aeruginosa* PAO1 H103 was provided by the Hancock Lab (Department of Microbiology and Immunology, University of British Columbia). Mutant strains PAO1_lux_81_G7 and PAO1_lux_22_A5 were generated as previously described (16). Annexin V-biotin was purchased from Sigma (Cat. no. A7810). Unless otherwise indicated, all chemicals were purchased from Sigma. HRP-streptavidin was purchased from PIERCE (Cat. no. N1000). Rabbit polyclonal anti-caspase-3 antibody was purchased from BD Pharmingen (Cat. no. 51-9000064). Jurkat cell lysate (camptothecin-treated) was purchased from BioVision (Cat. no. 2402-100).

Growth conditions. For overnight cultures, bacteria were grown for 16 h at 37 °C with aeration and agitation of 200 rpm. Four different liquid growth media were used: Luria-Bertani medium (LB) and M9 media with and without glycine and Starch Minimal media. LB media (pH 7.0) contained tryptone (1%, BD Science), yeast extract (0.5%, BD Science), and NaCl (1%). M9 media (pH 7.2) contained Na_2HPO_4 (0.6%), KH_2PO_4 (0.3%), NaCl (0.05%), NH_4Cl (0.1%), $MgSO_4 \cdot 7H_2O$ (1 mM), $CaCl_2$ (0.1 mM), glucose (2%), and trace elements solution ($FeCl_3$, $MnSO_4$, $ZnSO_4$, $CaSO_4$, H_3BO_3, Na_2MoO_4, $COCl_2$). For M9 with glycine, 80 mM of glycine (BD Science) was added after autoclaving before inoculation. Starch Minimal media (pH 7.0) contained starch (1%), K2PO4 (0.3%), KH2PO4 (0.15%), ammonium sulfate (0.2%), L-methionine (0.05%), L-glutamate (0.025%), and trace elements (as previous).

Biofilm formation assay. Overnight-grown *P. aeruginosa* PAO1 or mutant strain cells were diluted 1/10 in M9 media and transferred to different media types as indicated in 96-well polystyrene plates (BD Biosciences) for a total dilution of 1/1000 and eight replicates of 100 µl total volume. Cultures continued to grow at 37 °C in a humidified 5% CO_2 incubator. At indicated times, cultures were removed and the wells were washed twice with 150 µl of phosphate-buffered saline (PBS2; 10 mM, pH 7.5, Gibco). Remaining cells were incubated with 150 µl of a 1% crystal violet solution in 95% methanol for 15 min at room temperature, followed by two washes with PBS. Pure Dimethyl Sulfoxide (DMSO) was added to the crystal violet stained cells to absorb the dye for 5 min, and this was transferred to new wells for absorbance at 595 nm [A_{595}] readings (7).

SDS-PAGE. Overnight-grown *P. aeruginosa* PAO1 cells were diluted 1/10 in M9 media and transferred to different media types as indicated in a 12-well polystyrene plate for a total dilution of 1/1000. At indicated times, cultures were removed and the wells were washed twice with phosphate-buffered saline (PBS2; 10 mM, pH 7.5). Remaining cells were lysed directly using 2% Triton X-100 lysis buffer with protease inhibitors (2x lysis buffer (50 mM Tris-HCl (pH 7.2), 2% Triton X-100, 60 mM KCl, 2 mM EDTA, 0.5 mM Na_3VO_4, 0.25 mM Na_2MoO_4, 0.25 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) and mechanical manipulation with the end of the pipette. Samples were mixed with equal volume of x 2 sample buffer (0.5 M Tris [pH 6.8], 20% sodium dodecyl sulfate [SDS] (EB Science), 40% glycerol, 0.1% bromophenol blue ß-mercaptoethanol [600 mM]). Samples were then heated to 95°C for 10 min, immediately chilled on ice for 5 min, vortexed on high speed for 1 minute, and centrifuged at 12,000 x *g* for 10 min before storing at -80 °C. Protein concentration was normalized by adjusting the volume of sample loaded according to the estimated relative concentration by biofilm assay. Samples were loaded on a 10% (wt/vol) Tris-glycine SDS-polyacrylamide gel (Biorad), which was run vertically at 120V on a Biorad polyacrylamide gel electrophoresis (PAGE) system.

Western blotting. After completion of the SDS-PAGE run, electroblotting was performed using a Hybond-P membrane (Amersham, RPN303F) in a transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% methanol) employing a 110V constant current for 45 min at room temperature. Protein detection was determined by Ponceau S stain (0.1% wt/vol in 5% acetic acid) before membrane was blocked using 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween 20 (0.05%) for 1 h at room temperature. The blotted membrane was incubated overnight with anti- human caspase-3 antibody in a 1/1000 dilution TBS-T with 1% BSA at 4 °C. After six 10 min washes in TSB-T, the anti-caspase antibody was subjected to secondary hybridization with horseradish peroxidase conjugated anti-rabbit antibody in a 1/10,000 dilution of TBS-T with 1 % BSA for 1 h at room temperature. The blot was washed six times with Tris-buffered saline (TBS)-Tween 20 (0.05%), once with TBS for 5 min, and detected using ECL (Amersham RPN2108).

Confocal microscopy. Overnight-grown *P. aeruginosa* PAO1 or mutant strain cells were diluted 1/10 in LB media and transferred to 8-chamber polystyrene slides with 1.5 µm thickness (ibidi Integrated BioDiagnostics Cat. no. 80826) for a total dilution of 1/1000 and 500 µl total volume over a 1 cm^2 area. Cultures continued to grow at 37 °C in a humidified CO_2 incubation with gentle rocking. After 24 h growth, media was changed every 4 h. At 60 h, biofilms were washed twice with 150 µl phosphate-buffered saline (PBS; 10 mM, pH 7.5, Gibco) and fixed with 4% paraformaldehyde for 25 min. Biofilms were subsequently permeabilized with 1.0% Triton X-100 in PBS and stained with fluorescein isothiocyanate (FITC) (Sigma) at 0.25% w/v in Tris-HCl pH 8.5 solution. Imaging was acquired using a 60x Plan Apochromat objective (NA, 1.35) of an Olympus FV1000 confocal microscope.

Congo red assay. LB-broth supplemented with congo red (40 µg/ml, Kodak) and coomassie brilliant blue (15 µg/ml, Kodak) were used to 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 °C to assess colony morphology.

Caspase-3 activity assay. Bacteria were grown overnight or isolated from biofilm formation assay conditions grown in 6-well polystyrene plates (BD Biosciences) and centrifuged at 5000 x *g* for 10 min. The pellet was washed with phosphate-buffered saline (PBS; 10 mM, pH 7.5, Gibco) and suspended in 250 µl lysis buffer [Tris-HCl (10 mM), NaH_2PO_4/$NaHPO_4$ (10 mM, pH 7.5), NaCl (130 mM), Triton X-100 (1%)]. The lysate was then sonicated with a Braun-Sonic 2000 sonicator (Ultrasonic Power Corp., Freeport, Ill.) using a microprobe at 60 watts for 10 s. Lysates were placed on ice for 30 min. A 100 µl aliquot of the cell lysate was reacted with 15 µl (1 mg/ml) of synthetic fluorogenic substrate (BD Pharmingen, Cat. no. 556449), N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin) at 37°C for 1 h in 1 ml reaction buffer (20 mM HEPES (pH 7.5); 10% glycerol; 2 mM DTT). For inhibitor addition reaction samples, 15 µl of 0.1 mg/ml of the synthetic inhibitor of caspase-3 (Ac-DEVD-CHO, BD Pharmingen Cat. no. 556465) was also added in addition to substrate and reaction buffer. After incubation, the fluorescence intensity was measured using the Perkin-Elmer Fluorescence Spectrophotometer Model 651-10S (excitation 380nm, emission 440 nm, and bandwidth 15 nm).

Motility assay. Swimming motility was evaluated on LB plates containing 0.3% agar, and swarming on LB plates containing 0.5% agar. Swimming and swarming colony formation were evaluated by seeding 1 µl aliquot of an overnight culture grown on the respective plates, and incubating for 24 h at 37 °C. Twitching was measured on LB plates containing 1.0% agar. Colonies were stab inoculated through the agar and incubated for 24 h at 37 °C.

Annexin V assay. Bacteria were grown overnight or isolated from the biofilm formation assay conditions grown in 6-well polystyrene plates (Falcon) and centrifuged at 5,000 x *g* for 10 min. The pellet was washed twice with ice cold phosphate-buffered saline (PBS; 10 mM, pH 7.5, Gibco). Washed cells were incubated with a 1/500 dilution of Annexin V-biotin in PBS for 1 h at 4 °C followed by centrifugation at 10,000 x *g* for 2 min. Cells were subsequently washed three times with PBS followed by centrifugation at 10,000 x *g* for 2 min and then incubated with HRP-streptavidin for 1 h at 4 °C. Cells were again washed and incubated with 750 µl 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma, T-8665), followed by 750 µl of a stop solution (0.18 M H_2SO_4) and absorbance readings were taken at 450 nm [A_{450}].

RESULTS

Upregulation of caspase-3 like proteins with biofilm growth in *Pseudomonas aeruginosa*. Several groups have determined a nutritional requirement for structured biofilm formation dependent on amino acids and carbon source (25, 22). In order to determine conditions for robust biofilm formation, we used different media types and carried out a time course evaluation of biofilm formation by measuring adherent biomass. Consistent with other findings, minimal media support little biofilm growth, which is slightly increased with the addition of glycine (Fig. 1A). Here, we wanted a system to test more robust biofilms and used LB cultures to determine the presence of a caspase-3 like protein by western blot. This expression was time dependent and correlated with early stages of biofilm development. Two molecular weight bands of 85 and 60 kDa were observed (Fig. 1B), both of which showed little expression in an overnight culture and decreased expression at 60 h in polystyrene 96-well microtitre plates. A positive control of Jurkat T cells treated with camptothecin, which induces apoptosis in eukaryotic cells, showed human caspase-3 expression corresponding to an expected 30 kDa.

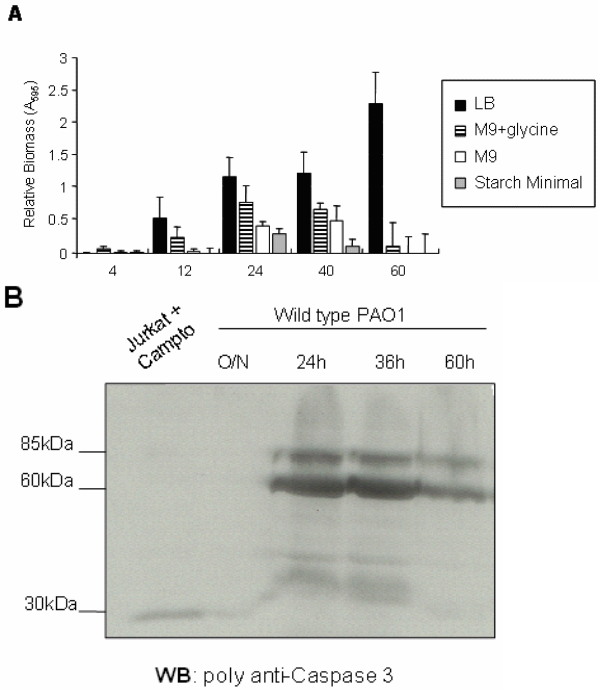

FIG. 1. (A) Nutrient requirement for biofilm formation by *Pseudomonas aeruginosa*. Overnight PAO1 culture was used to inoculate indicated media types for growth at 37 °C in 96-well polystyrene microtitre plates. Surface-associated biofilm formation was analysed by crystal violet staining of the adherent biofilm followed by the extraction of the crystal violet with DMSO and measurement of A_{595}. Means±SD of three replicates are plotted. (B) Inducible expression of a capase-3 like protein in *P. aeruginosa* biofilm. Overnight PAO1 culture was either lysed or used to inoculate LB media for growth in polystyrene plates. Planktonic cells were removed and remaining biomass was normalized by A_{595} in part (A). A positive control of camptothecin treated Jurkat T cell lysate was included as positive control.

Identification of a caspase-3 like protein, Clp, and *clp* : : *lux* phenotype. Given the cross-reaction of an anti-human caspase-3 antibody to induced proteins in *P. aeruginosa* biofilms, we used the amino acid sequence of the caspase-3 active site to BLAST within the mini-Tn*5-luxCDABE* fusion PAO1 library (16) using operon finding software V1.2 (http://v2.pseudomonas.com/). We identified three potential matches: a probable ATP-dependent protease, a probable peptidase, and a hypothetical protein (Supplemental Table 1). Given the predicted molecular weight of 88 kDa and high homology to LonB in *E. coli*, we further investigated the PA4576 gene (Fig. 2A), a probable ATP-dependent protease. PA4576 has nearly 99% alignment to the LonB consensus sequence between aa48-790, but interestingly only 28% alignment to the C-terminus of Lon in *Pseudomonas*

(Supplemental Fig. 1). The two other matches had predicted 67 kDa and 34 kDa molecular weights, and may correspond to the other observed bands. The colony morphology of both *clp* : : *lux* mutants for the PA4576 gene showed numerous voids on both minimal and rich media, more frayed edges, and reduced thickness of the colony as observed with light field images in the lower right corners (Fig. 2B). Interestingly, the ClpΔ672-817 phenotype is less severe than the ClpΔ517-817, though both mutants do not contain the putitive active site for the protease. Images are representative of three independent experiments. Phenotypic differences were first observed <72 h post seeding (data not shown).

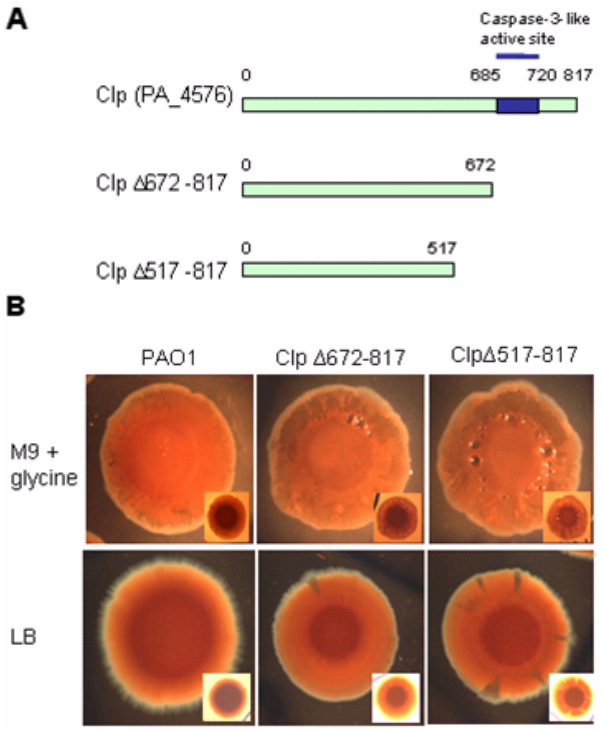

FIG. 2. (A) Identification of a caspase-3 like protease in the PAO1 *lux* library. Amino acid sequence of a eukaryotic caspase-3 active site showed high homology to the C-terminus of the PA4576 gene product. The two mutant forms from the *lux* library are shown. (B) *clp* : : *lux* colony morphology as shown by using a Congo Red assay. Overnight cultures were normalized by OD_{460} and plated. Images shown represent 6 day phenotype.

Clp is required for normal biofilm formation. To determine if Clp was important in the normal biofilm formation of *P. aeruginosa*, we used the two *clp* : : *lux* mutants in a biofilm formation assay and measured adherent biomass (Fig. 3A). Both mutants showed a significant decrease in early stages of biofilm formation, which was sustained at later time points for the ClpΔ517-817 mutant. In order to further characterize the biofilm structure of *P. aeruginosa* and *lux* mutants, we used confocal microscopy to detail the thickness and complexity of the formed biomass. Using surface and sideview projections, *clp* : : *lux* mutants completely do not establish channels, are reduced 5 fold in thickness, and are flat (Fig. 3B). FITC staining is more intense in the PAO1 biofilms and channels are consistent throughout (Supplemental Movies 1 and 2). The ClpΔ672-817 showed an increased thickness, but similar complexity to the ClpΔ517-817 mutant (data not shown).

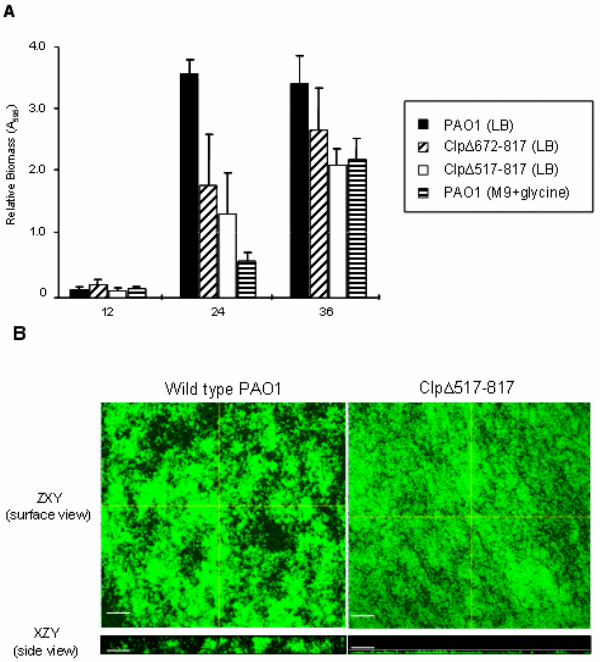

FIG. 3. (A) Biofilm formation by *P. aeruginosa* PAO1 and *clp* : : *lux* fusions. Bacteria were grown in 96-well polystyrene microtitre plates containing LB and surface-associated biofilm was analysed by crystal violet staining as measured by A_{595}. (B) Confocal microscopy of PAO1 and *clp* : : *lux* biofilm structure. Bacteria were grown at 37 °C in 8-chamber polystyrene slides with gentle rocking for 24 h followed by media replacement every 4-6 h for 36 h. Biofilms were washed, fixed, and stained with FITC. Images are representative *z*-projections with *x*-*z* projection shown below. Size bars represent 15 *u*m.

Clp is not required for normal swimming, swarming, or twitching. Given the dramatic loss of biofilm structure observed in *clp* : : *lux* mutants, we investigated the motility of these mutants. Our previous work using a *lon* : : *lux* mutant has shown the Lon protease in *P. aeruginosa* is not required for the normal presence of pili but is required for normal motility, indicative of a role in flagellum activity (20). Similar motility assays were carried out using the *clp* : : *lux* mutants, which showed no decrease in colony diameter on 0.3 % and 0.5 % agar (Fig. 4). Mutants also showed no difference in a twitching assay, suggesting that Clp has a different function from Lon in normal biofilm formation.

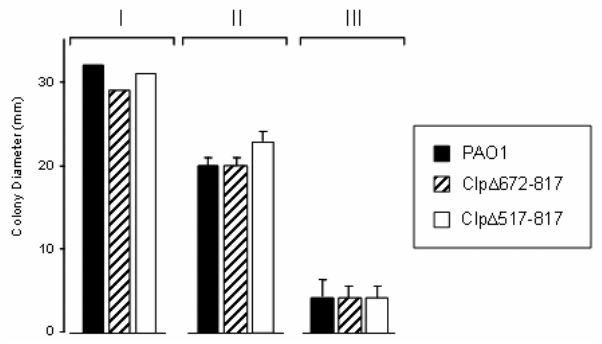

FIG. 4. Swimming (I), swarming (II) and twitching (III) motility of *P. aeruginosa* PAO1 and *clp* : : *lux*. For swimming and swarming, 1µl of overnight culture was spot-inoculated onto LB swarm plates containing 0.3 % and 0.5 % agar, respectively. Twitching was measured by stab-inoculation through LB plates containing 1.0 % agar. Colony diameters were measured after 22 h incubation at 37 °C. Means and Means±SD of three experiments are plotted.

The modulation of rapid cell death by Clp. In order to characterize this unique function, *clp* : : *lux* mutants were used in a caspase-3 activity assay. Biofilm cultures have a 50% increase in caspase activity compared with overnight cultures, which was completely lost in the ClpΔ517-817 mutant. As with results obtained in other assays, the ClpΔ672-817 mutant showed a less significant impairment. Biofilm and overnight cultures were normalized by OD_{460} and values were normalized using a non-substrate control. The caspase-3 inhibitor, Ac-DEVD-CHO, did not affect these results (Fig. 5A). At later time points, there was an observable increase in measured caspase activity of the mutants, suggesting that subsequent stages of biofilm formation may involve other caspase-like proteases (data not shown). To further describe the biological significance of this activity, we used an Annexin V assay to measure the exposed phosphatidylserine (PS) residues on the outer membrane of the Gram-negative bacteria. PS is found in trace amounts in Gram-negative bacteria in both inner and outer membranes (9, 15), but only exposed during cell death (27). Biofilm culture showed an increase in exposed PS residues as compared to overnight culture (Fig. 5B). Both *clp* : : *lux* mutants however, did not show this increase. This difference was less dramatic at 24 h, but showed the same trend (data not shown).

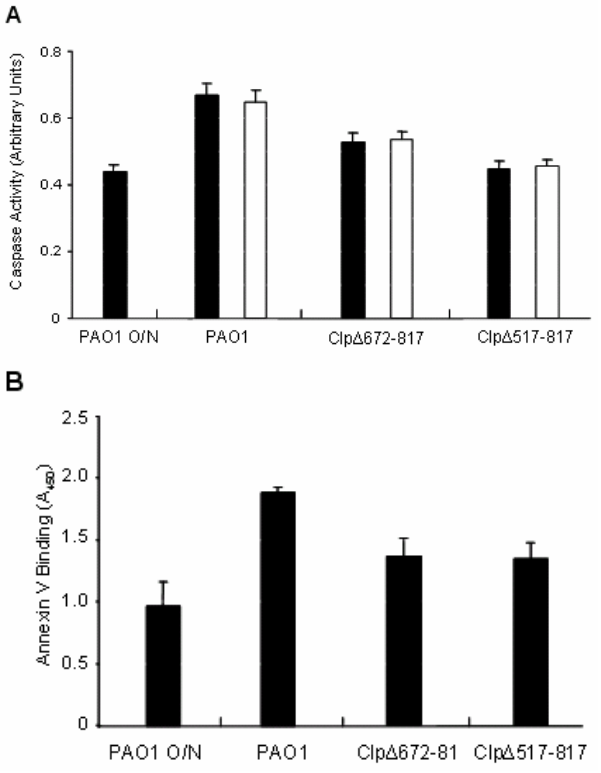

FIG. 5. (A) Caspase activity of *P. aeruginosa* PAO1 and *clp* : : *lux* biofilm. Caspase-3 activity was determined using fresh cell lysate from overnight or biofilm cultures in a reaction mixture containing the synthetic fluorogenic substrate (Ac-DEVD-AMC) of caspase-3 with (■) or without caspase-3 inhibitor (□) and measuring the fluorescence intensity. (B) Annexin V apoptotic assay. Cells from overnight and biofilm cultures at 36 h were washed and incubated with biotinylated Annexin V. The samples were then reacted with HRP-conjugated streptavidin and TMB substrate and stopped using a 0.1 M final concentration of H_2S0_4. Absorbance readings were taken at 450 nm.

DISCUSSION

Cell death is known to be involved in *Pseudomonas aeruginosa* biofilm formation (30). Recent studies have also shown that caspase-3-like proteins may partly regulate bacterial rapid cell death (RCD) of *Xanthomonas campestris*, however, no caspase-like gene has been identified (8). In the current investigation, we confirmed caspase-3-like expression in *P. aeruginosa* biofilms, and further characterized this expression as inducible. Bioinformatic analysis of the human caspase-3 active site led to the investigation of the PA4576 gene, which shows high homology to the Lon protease family, specifically LonB. We refer to this gene in this study as *clp*, a caspase-3 like Lon protease.

We have determined Clp to be required for both nutrient limited growth and structured biofilm formation, assumed to be a survival strategy for nutrient limited micro-environment. Though others have shown biofilm formation of *P. aeruginosa* requires numerous motility genes such as *pilA* and *fliM* (22, 29), and genes which indirectly control motility including *lon* (20), our studies do not implicate *clp* in regulating motility. An alternative mechanism by which Clp mediates structured biofilm formation may be an apoptotic-like proteolytic cascade. Evidence of

phosphatidylserine exposure on the outer leaflet of the outer membrane which is reduced in both *clp* : : *lux* mutants and overnight culture is consistent this apoptotic model.

Interestingly, a caspase activity assay was able to detect cleavage by Clp in biofilms, which was not evident in overnight culture. This enzyme activity during biofilm establishment stages appeared to be exclusively Clp mediated, and required the complete C-terminus of the protease. How proximal domains affect this activity are unclear, though in comparing our two *clp* : : *lux* mutants, it is evident that Clp may either be in a complex or have downstream substrates which directly cleave this assayed substrate based on the discrepancy between different truncations of the C-terminus. Later biofilm formation time points have not been thoroughly investigated. However, preliminary data suggests that other proteases are able to cleave this substrate at 48 h (R. Castaneda & G. X. Song-Zhao, unpublished data).

Current models for bacterial autolysis in *P. aeruginosa* implicate quorum sensing signals leading to the activation and release of endogenous Pfl-like prophage (4, 29). Other researchers have suggested an ancestral apoptotic system in bacteria which evolved into classic eukaryotic apoptosis involving caspase cascades (12). Several proteins known to be involved in apoptosis have been compared to bacterial genomes, and many similar protein domains identified (12). Yet other models correlate toxin-antitoxin (TA) systems with autolysis, which also require proteolytic cleavage of antitoxin components (17). In *E. coli*, it has been shown that Lon degrades the unstable antitoxins of both the *relBE* and *mazEF* TA systems, thereby allowing respective toxins to act as translation inhibitors (2, 3). It has been postulated that activation of the *mazEF* system during amino acid starvation is a means of initiating rapid cell death (1).

Both *lonA* and *lonB* genes are found in the *lon-clpx* operon in *B. subtilis* (18) however, in *Pseudomonas aeruginosa*, *Clp* is not proximal to either *lon* or *clpx*. LonB studies in *B. subtilis* have shown its presence during sporulation in the forespore complex, and suggested requirement for a second unknown protein to become an active protease (29). The mechanisms by which LonB expression and activity are regulated in the Gram-positive *Bacillus* may elucidate an attractive comparison between the nutrient deprived response of sporulation and the Gram-negative response of autolysis during structured biofilm formation.

Based on our observations and current understanding of bacterial autolysis, we speculate that Clp is required for autolysis leading to structured and robust biofilms with channels. The proteolytic activity of Clp may initiate a cascade that ultimately leads to the selective killing of a subset of cells inside the biofilm. If *P. aeruginosa* does undergo a programmed and controlled cell death pathway, this would be an interesting target for anti-biofilm therapy.

FUTURE EXPERIMENTS

Initial studies into Clp should include using Western Blot analysis of *clp :: lux* mutants for caspase expression which is part of our continued investigation. To further elucidate mechanisms for the role of Lon proteases in autolysis, the *Pseudomonas aeruginosa* genome could be examined for the presence of toxin-antitoxin systems through bioinformatics. This would involve identification of the known TA systems involving Lon protease of other organisms and BLASTing them against the *Pseudomonas aeruginosa* genome. Subsequently, the mutants of these TA systems could be evaluated for their phenotype in biofilm formation. Secondly, to distinguish the functions between Clp and the existing Lon gene in *Pseudomonas aeruginosa* (PA1803), the knock-out mutants of both of these genes should be compared for any observable phenotypic differences. Also, BacLight LIVE/DEAD viability probe (Molecular Probes) should be used in confocal microscopy to gain more insight into the localization of autolysis in bacterial biofilms (30). Important studies which determine variations on caspase inhibitors to be permeable to Gram negative cells and specific to Clp should be carried out, as these will potentially lead to anti-biofilm therapies, however, the mechanisms by which Clp and other proteases work in rapid cell death must first be understood.

ACKNOWLEDGEMENTS

This work is supported by the funds from the Microbiology and Immunology department of the University of British Columbia, specifically for the Experimental Microbiology 421 course. We would like to thank Dr. Robert Hancock, W.J. Gooderham and Susan Farmer for their assistance with the mini-Tn*5*-*luxCDABE* fusion PAO1 library and generous gift of *P. aeruginosa* strains. We also express our appreciation to Dr. John Smit and Dr. Mike Gold for the use of lab equipment. We would like to send special thanks to Marcia Graves for imaging expertise and guidance. We would also like to express our gratitude to Jennifer Sibley, Lando Robillo, John Nomellini, Manjeet Bains, Eileen Hinze, Karen Smith and Dr. William Ramey for their assistance and support throughout this project.

REFERENCES

1. **Aizenman, E., H. Engelberg-Kulka, and G. Glaser.** 1996. An *Escherichia coli* chromosomal "addiction module" regulated by guanosine 3',5'-bispyrophosphate: A model

for programmed bacterial cell death. Proc. Natl. Acad. Sci. **93:**6059-6063.

2. **Christensen, S.K., M. Mikkelsen, K. Pedersen, and K. Gerdes.** 2001. RelE, a global inhibitor of translation, is activated during nutritional stress. Proc. Natl. Acad. Sci. **98:**14328-14333.
3. **Christensen, S.K., K. Pedersen, F.G. Hansen, and K. Gerdes.** 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNA's and are counteracted by tmRNA. J. Mol. Biol. **332:**809-819.
4. **D'Argenio, D.A., M.W. Calfee, P.B. Rainey, and E.C. Pesci.** 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. J. Bacteriol. **184:**6481–6489.
5. **Davey, M. E., and G.A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847-867.
6. **Drenkard, E.** 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microb. Infect. **5:**1213-1219.
7. **Friedman, L., and R. Kolter.** 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol. Microbiol. **51:**675-690.
8. **Gautam, S., and A. Sharma.** 2002. Involvement of caspase-3-like protein in rapid cell death of *Xanthomonas*. Mol. Microbiol. **44:**393-401.
9. **Istivan, T.S., and P.J. Coloe.** 2006. Phospholipase A in gram-negative bacteria and its role in pathogenesis. Microbiol. **152:**1263-1274.
10. **Kirisits, M.J., and M. Parsek.** 2006. Does *Pseudomonas aeruginosa* use intercellular signalling to build biofilm communities? Cell Microbiol. **8**:1841-1849.
11. **Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen.** 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol. Microbiol. **48**:1511-1524.
12. **Koonin, E.V., and L. Aravind.** 2002. Origin and evolution of eukaryotic apoptosis: the bacterial connection. Cell Death Differ. **9:**394–404.
13. **Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, and L.T. Wiliams.** 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science. **278:**294-298.
14. **Lack, N., R. Liem, R. Verhulp, and L. Willinghanz**. 2004. Evidence for a putative caspase-3-like protein in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. J. Exp. Microbiol. Immunol. **5**:16-22.
15. **Langley, K.E., E. Hawrot, and E.P. Kennedy.** 1982. Membrane assembly: movement of phosphatidylserine between the cytoplasmic and outer membranes of *Escherichia coli*. J. Bacteriol. **152:**1033-1041.
16. **Lewenza, S., R.K. Falsafi, G. Winsor, W.J. Gooderham, J.B. McPhee, F.S.L. Brinkman, and R.E.W. Hancock**. 2005. Construction of a mini-Tn5-luxCDABE mutant library in Pseudomonas aeruginosa PAO1: A tool for identifying differentially regulated genes. Genome Res. **15**:583-589.
17. **Lewis, K.** 2000. Programmed death in bacteria. Microbiol. Mol. Biol. Rev. **64:**503–514.
18. **Liu, J., W.M. Cosby, and P. Zuber.** 1999. Role of Lon and ClpX in the post-translational regulation of a sigma subunit of RNA polymerase required for cellular differentiation in *Bacillus subtilis*. Mol. Microbiol. **33:**415-428.
19. **Mai-Prochnow, A., F. Evans, D. Dalisay-Saludes, S. Stelzer, S. Egan, S. James, J.S. Webb, and S. Kjelleberg.** 2004. Biofilm development and cell death in the marine bacterium *Pseudoalteromonas tunicata*. Appl. Environ. Microbiol. **70**:3232-3238.
20. **Marr, A.K., J. Overhage, M. Bains, and R.E.W. Hancock.** 2007. The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. Microbiol. **153:**474-482.
21. **Meluleni, G.J., M. Grout, D.J. Evans, and G.B. Pier.** 1995. Mucoid *Pseudomonas aeruginosa* growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. J. Immunol. **155:**2029-2038.
22. **O'Toole, G., H.B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. **54:**49-79.
23. **O'Toole, G.A., K.A. Gibbs, P.W. Hager, P.V. Phibbs, Jr, and R. Kolter.** 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. J. Bacteriol. **182:**425-431.
24. **Pamp, S.J., and T. Tolker-Nielsen.** 2007. Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. J. Bacteriol. **189**:2531-2539.
25. **Raju, K.K., S. Gautam, and A. Sharma.** 2006. Molecules involved in the modulation of rapid cell death in *Xanthomonas*. J. Bacteriol. **188**:5408-5416.
26. **Rice, K.C., and K.W. Bayles.** 2003. Death's toolbox: examining the molecular components of bacterial programmed cell death. Mol. Microbiol. **50:**729-738.
27. **Ruiz, N., D. Kahne, and T. J. Silhavy**. 2006. Advances in understanding bacterial outer-membrane biogenesis. Nat.Rev.Microbiol. **4**:57-66.
28. **Sauer, K., A.K. Camper, G.D. Ehrlich, J.W. Costerton, and D.G. Davies.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. **184:**1140-1154.
29. **Serrano, M., and U. Volker.** 2001. Forespore-specific transcription of the *lonB* gene during sporulation in *Bacillus subtilis.* J. Bacteriol. **183:**2955-3003.
30. **Webb, J.S., L.S. Thompson, S. James, T. Charlton, T. Tolker-Nielsen, B. Koch, M. Givskov, and S. Kjelleberg.** 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. J. Bacteriol. **185**:4585-4592.