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Evidence for a Putative Caspase-3-Like Protein in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*

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The process of apoptosis, or programmed cell death, involves proteolytic cascades mediated by caspase proteins. One protein that plays a central role in the induction of apoptosis, is caspase-3, a cysteinyl aspartate-specific protease. Although apoptosis is often characterized as a eukaryotic process, recent studies have demonstrated evidence for prokaryotic programmed cell death and have identified the presence of a caspase-3-like protein in *Xanthomonas campestris*. Therefore, in this study we tested for the presence of a caspase-3-like protein in a broad range of bacteria, including *Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Bacillus subtilis,* and *Escherichia coli* strains. We found evidence for both the expression of a caspase-3-like protein, as well as caspase-3 enzymatic activity in both *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. However, due to the preliminary nature of these studies, additional experiments are necessary to conclude that the presence and activity of a caspase-3-like protein are directly related to the process of apoptosis in prokaryotes.

Apoptosis, also known as programmed cell death (PCD), is an active process employed by eukaryotic cells during embryonic development and in the maintenance of adult tissues in multicellular organisms (4). The distinct morphological and functional changes characteristic of apoptosis are mediated by caspase proteins, an instrumental family of aspartate-dependent cysteine proteases, which play a central role in both the initiation and the activation of apoptosis. Initiator caspases, such as caspase-8, are known to activate downstream effector caspases, including caspase-3 (also known as CPP32, Yama, and Apopain) (25). Caspase-3 is initially synthesized in an inactive pro-enzyme form (28), which is subsequently processed into two subunits by self-proteolysis or cleavage by an upstream caspase (13). These subunits form the active enzyme which proteolytically cleaves and activates other caspases, as well as relevant targets in the cells, including: cytoskeleton proteins such as gelsolin (14), regulators of DNA repair (e.g. poly ADP-ribose polymerase) and cell cycle proteins (2). Although apoptosis has mainly been identified in eukaryotic organisms, there is increasing evidence for the presence of prokaryotic PCD (1, 11, 17, 18, 22, 23, 27). In fact, bacterial PCD has been shown to occur in *Xanthomonas campestris*, a pathogen which infects soybean plants (8), through a caspase-3 dependent pathway (10). In that study, the presence and the enzymatic activity of a caspase-3-like (C3L) protein were shown to correlate with classical hallmarks of apoptosis, including the binding of Annexin-V to the cell membrane and the presence of nicked DNA (10).

The formation of biofilms is fundamental to the survival of a broad range of microorganisms in diverse environmental, industrial and clinical settings (6). Biofilm formation in *Pseudomonas aeruginosa* has been shown to correspond to both the development of antibiotic-resistant infections in cystic fibrosis patients (3, 9) and the survival of a bacterial population against the host immune response (16). *Stenotrophomonas maltophilia* (previously identified as *X. maltophilia* (21) is known to form biofilms associated with implant-associated infection through the colonization of synthetic surfaces, such as catheters (7). Within biofilms, it has been suggested that apoptosis may be crucial for survival during times of limited nutrient supply (22). This hypothesis proposes that the death of the bacteria in the center of the biofilm would increase the chance of survival for the whole bacterial population. Accordingly, those bacteria which could form biofilms, including *S. maltophilia* and *P. aeruginosa,* would have the ability to induce apoptosis in sub-optimal conditions.

In this study, we examined *S. maltophilia* H582 and H361; *P. aeruginosa* H829, H823, and H822; *Bacillus subtilis* JH642; and *E. coli* DH5α for the presence and the activity of a caspase-3-like protein. Specifically, we focused on whether the expression and the activity of such a protein is induced by suboptimal growth conditions that have been suggested to induce apoptosis. A C3L protein was detected in *B. subtilis* JH642, *S. maltophilia* H361, and *P. aeruginosa* H829, H823, and H822. Furthermore, enzymatic assays demonstrated C3L activity in *S. maltophilia* H361 and in *P. aeruginosa* H823.

MATERIALS AND METHODS

Strains and chemicals. *S. maltophilia* H582 (24), *S. maltophilia* H361 (ATCC# 136737), *P. aeruginosa* H829, *P. aeruginosa* H823, and *P. aeruginosa* H822 were kindly provided by Susan Farmer of the Bob Hancock Laboratory (*S. maltophilia* strains were initially identified as *X. maltophilia*; all *S. maltophilia* and *P. aeruginosa* strains originated as clinical isolates). *Bacillus subtilis* JH642 and *E. coli* DH5α were generously donated by the George Spiegelman Laboratory.

Growth conditions. Bacteria were grown overnight with aeration in the conditions shown in Table 1. Luria-Bertani medium (LB) contained 1% peptone, 0.5% yeast extract, and 0.5% sodium chloride. Starch medium contained 1% starch, 0.3% $K_2HPO_4 \cdot 3H_2O$, 0.15% KH_2PO_4, 0.2% ammonium sulphate, 0.05% L-methionine, 0.025% nicotinic acid, and 0.025% L-glutamate, at pH 6.8. Pro-lytic medium contained 0.5% tryptone medium, 1% glucose, 0.5% sodium chloride, and 0.5% L-arginine. Shaeffer's Sporulation media (SSM) contained 0.8% Nutrient broth, 0.0125% $MgSO_4 \cdot 7H_20$, 0.05% KCl, 1 µM $FeSO_4$, 10 µM $MnCl_2 \cdot 4H_20$, 1 mM $CaCl_2 \cdot 2H_20$, and 10 mg/ml each trytophan and phenylalanine, at pH 7.5.

TABLE 1. Standard and variant growth conditions for strains

Strain	Media	
	Standard[a]	Stressed[b]
S. maltophilia H582	Starch	LB
S. maltophilia H361	Starch	LB
P. aeruginosa H829	LB	Pro-lytic
P. aeruginosa H823	LB	Pro-lytic
P. aeruginosa H822	LB	Pro-lytic
B. subtilis JH642	LB	SSM
E. coli DH5α	LB	Starch

[a]Standard cultures were grown at 26ºC.
[b]Stressed cultures were grown at 37ºC.

Sample preparation. One ml samples were taken from each of the 14 cultures at 0, 4, 8, 18, and 24 hours; pelleted at 10,000 x g for 10 minutes; and frozen at -20°C. Pellets were washed twice with 1ml cold phosphate-buffered saline (PBS pH 7.4, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, 0.2 g/L KCl and 8 g/L NaCl) and resuspended in sterile distilled de-ionized water (10). For western blotting, cells were mixed with an equal volume of 2x loading buffer (0.1 M Tris pH 7.5, 1% SDS, 17.5% glycerol, 0.0125% bromophenol blue) and heated at 95°C for 5 minutes. For the enzyme assay, the cell lysis protocol was adapted from the methods of Gautam *et al.* (10) with the following changes: cell numbers were not normalized, and the lysis buffer contained 50 mM Tris-HCl pH 7.5, 1 mM DTT, and 0.3% NP-40 (19). In addition, the cells were sonicated at level 3 for approximately 1 minute, with a Misonix SonicatorXL (Farmingdale, NY).

Western blotting. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis under denaturing conditions and transferred onto Millipore Immobilon-P PVDF membranes (Millipore, Bedford, MA). The membranes were blocked for 1 hour in PBST (PBS with 0.1% [w/v] Tween-20) containing 5% [w/v] skim milk). Immunoblots were probed with rabbit anti-caspase-3 polyclonal antibody (1:200; MBL, Woburn, MA) for 1 hour followed by horseradish peroxidase-linked anti-rabbit immunoglobulin G whole antibody (1:10,000; Amersham Biosciences, Baie d'Urfé, QC) for 45 minutes. All wash steps to this point used PBST and all incubations were performed at room temperature. Membranes were then washed twice with PBS and detected using ECL Western Blotting Detection Reagent (Amersham Biosciences, Baie d'Urfé, QC) and Kodak Biomax Light X-ray film (Rochester, NY).

Caspase-3 assay. Synthetic tetrapeptide caspase-3 substrate Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) was obtained from Biosource International (Camarillo, CA). Assay samples of cell lysate were incubated with 0.2 mM Ac-DEVD-pNA for 21 hours at 37°C, and release of pNA was monitored at 405 nm with a BioRad-3550 spectrophotometer (15).

RESULTS

Growth of bacterial cultures. As shown in Figure 1, there was bacterial growth in all 14 cultures. The sizes of the cell pellets were recorded to indicate the relative amount of cells present in each sample. Pellet size was based on the following scale: 0 = no visible pellet, 1 = small pellet, 2 = medium pellet, 3 = large pellet. The 24 hour time point displayed the most pronounced growth, with all cultures containing pellets with a score of either 2 or 3. It is important to note that this scoring system provides only an estimate of the relative amount of bacteria per sample. The cultures in standard conditions were incubated at 26°C while the cultures in stressed conditions were grown at 37°C.

Western Blot analysis of bacterial C3L proteins. Through Western blot analysis using human polyclonal caspase-3 antibodies, we were able to demonstrate the presence of bacterial C3L proteins in six of the seven bacterial strains. As is evident from Figure 2, a strong hybridization signal was obtained for *S. maltophilia* H361-V.24, *P. aeruginosa* H829-S.24, H823-S.24, H823-V.24, H822-S.24, H822-V.24, and E. *coli* DH5α –S.24, while a weaker hybridization signal was seen in *P. aeruginosa* H822-V.0, H822-V.4, H829-V.24, *B. subtilis* JH642-S.4, JH642-S.8, JH642-S.24, and *E. coli* DH5α –S.8 *P*. There was no hybridization present in S. *maltophilia* H361 grown in standard conditions, *E. coli* DH5α grown in stressed conditions, and *S. maltophilia* H582 grown in either stressed or standard conditions.

In the S. *maltophilia* H361-V.24 sample, the molecular weights of the bands are approximately 50 kDa, 32 kDa and < 22 kDa. For the *P. aeruginosa* H823-S.24 sample, the molecular weights of the bands were approximately 55 kDa, 35kDa and < 22 kDa. For the *P. aeruginosa* H823-V.24, H823-S.24 and H823-V.24 samples, there was one 35kDa band present in all three samples, with *P. aeruginosa* H823-S.24 showing the strongest hybridization. The *E. coli* DH5α –S.24 sample showed a strong band present at < 22 kDa .

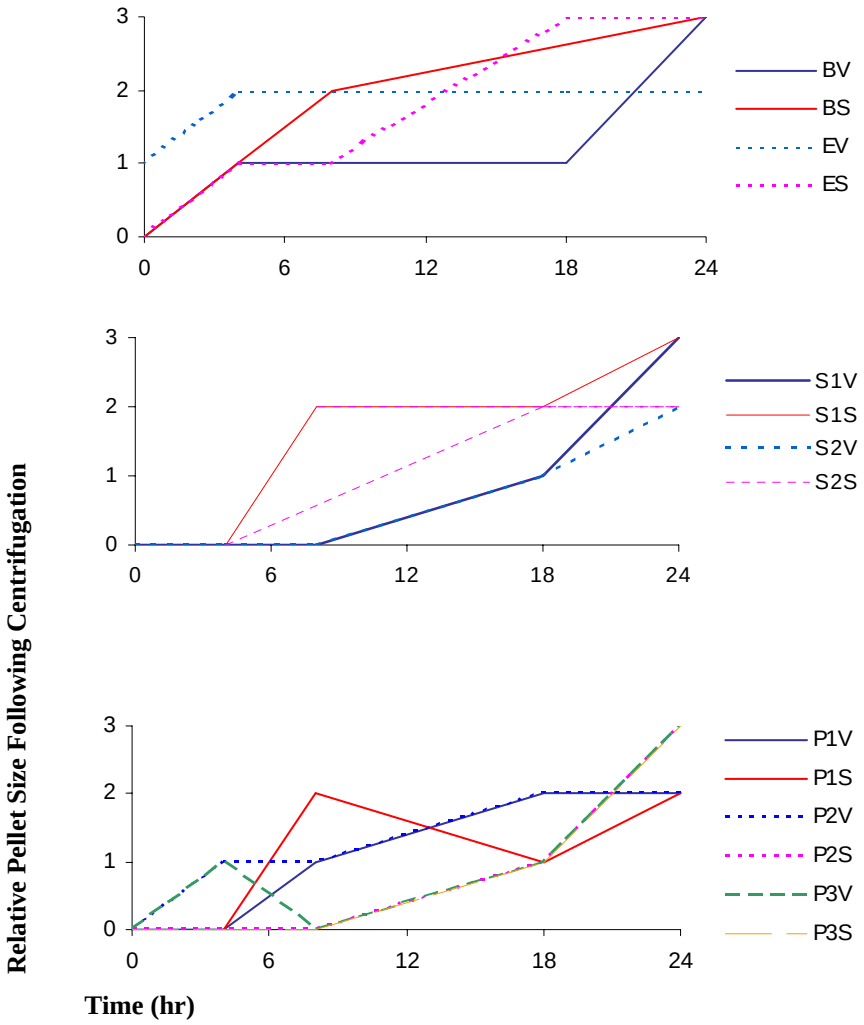

Figure 1. Relative size of pellets from culture samples obtained at various time points. Pellet size was based on the following scale: 0 = no visible pellet, 1 = small pellet, 2 = medium pellet, 3 = large pellet. The cultures grown in standard conditions were incubated at 26ºC while the stressed condition samples were grown at 37ºC. *S. maltophilia* H582 (S1), *S. maltophilia* H361 (S2), *P. aeruginosa* H829 (P1), *P. aeruginosa* H822 (P3), *B. subtilis* JH42 (B) and *E. coli* DH5α (E) were grown in either stressed (V) or standard (S) conditions.

Presence of caspase-3 activity. The 24 hour samples in the 14 different cultures were assayed using the chromogenic substrate of caspase-3, Ac-DEVD-AMC. Although a weak signal was detected in the *S. maltophilia* H361-V sample after 2 hours (data not shown), it was necessary to incubate the sample for 21 hours to measure the enzymatic activity. The values shown in Figure 3 are the standardized enzyme activity. These standardized values

were obtained by removing the average absorbance in the background controls from the sample readings. The background control contained enzyme substrate and lysis buffer with no cells.

DISCUSSION

Caspase-3, a cysteinyl aspartate-specific proteinase, is a central protein in programmed cell death and has been found to be conserved throughout various species, from man to nematode (28). In this study, various bacterial strains were assayed for both the presence of C3L proteins and C3L enzymatic functionality. Of the strains tested, only *S. maltophilia* H361 grown in variant media and *P. aeruginosa* H823 grown in standard media were found to have both the presence of a C3L protein as well as C3L enzyme activity. Several other strains including *P. aeruginosa* H829-S, H823-V, H822-S, H822-V and *E. coli* DH5α-S showed strong C3L hybridization by western blot, but no C3L enzymatic activity.

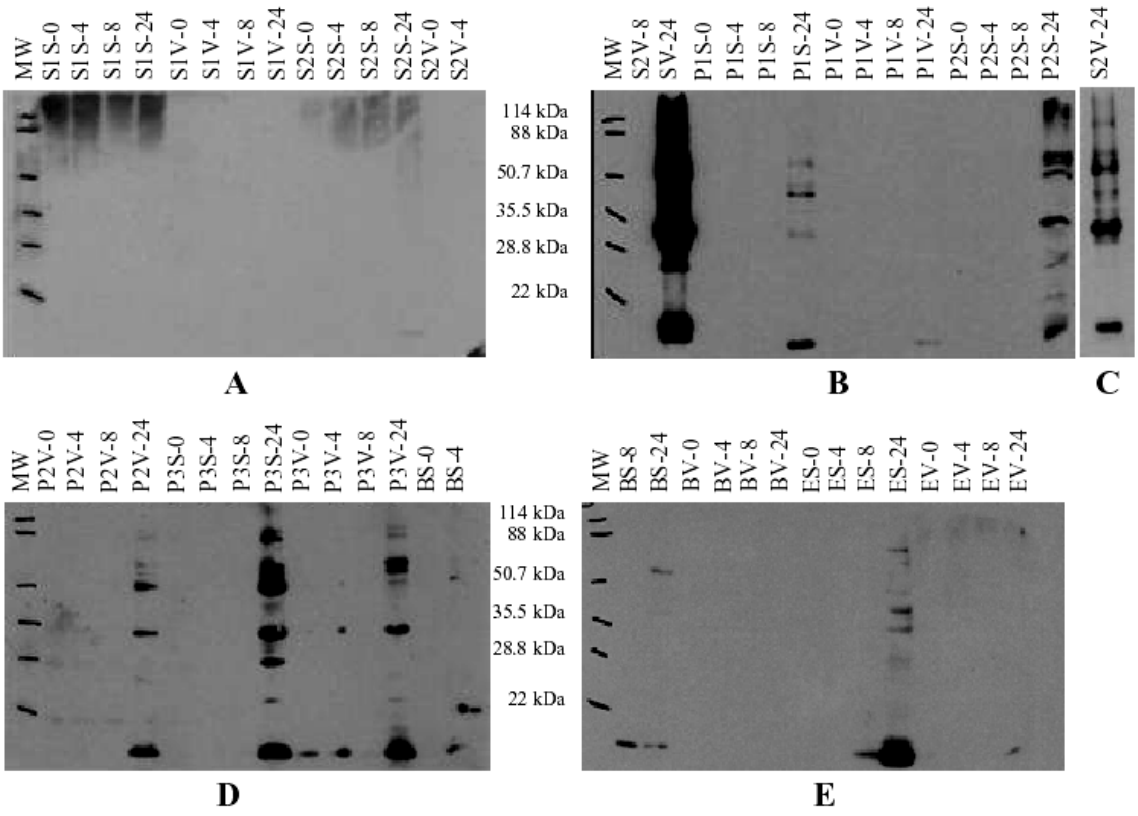

Figure 2. Western blot with anti-caspase-3 antibody for *S. maltophilia* H582 (S1), *S. maltophilia* H361 (S2), *P.aeruginosa* H829 (P1), *P.aeruginosa* H823 (P2), *P.aeruginosa* H822 (P3), *B. subtilis* JH642 (B) and *E. coli* DH5 (E) grown in either stressed (V) or standard conditions. The cultures were sampled at 0,4,8 and 24 hours. The exposure times are as follows: A = 5 minutes, B = 1 minute, C = 5 seconds, D = 5 minutes and E = 1 minute

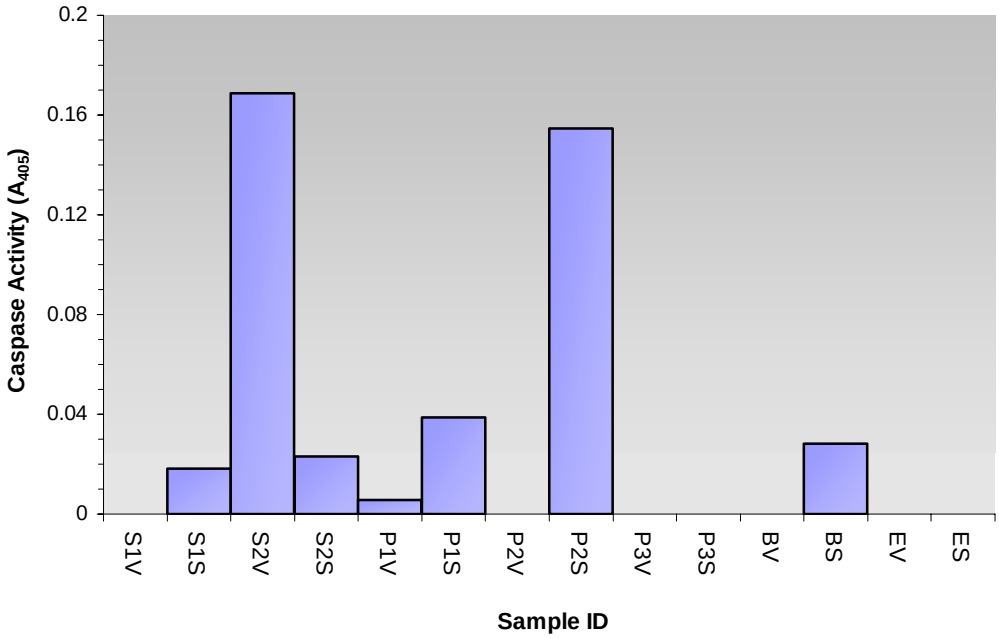

Figure 3. Standardized caspase activity of the 24 hour bacterial sample for *S. maltophilia* H582 (S1), *S. maltophilia* H361 (S2), *P. aeruginosa* H829 (P1), *P. aeruginosa* H823 (P2), *P. aeruginosa* H822 (P3), *B. subtilis* JH642 (B) and *E. coli* DH5 (E) grown in either Stressed (V) or Standard (S) conditions after 21 hour incubation with Ac-DEVD-AMC. Values are standardized by subtracting the background control values from the sample readings.

As the number of cells present in each pellet was not normalized, it is important that comparisons between samples are carefully interpreted. However, one should note that the extent of the presence of C3L protein in the western blot was not related to the size of the pellet, as the *S. maltophilia* H361-V sample which showed the strongest hybridization did not have the largest pellet. In examining the western blot of the 24 hour samples (Fig. 2), we see a clear trend emerging with the presence of a ~55 kDa "doublet". This banding pattern is similar to those results reported with *X. campestris* (10). As the polyclonal antibody is able to bind to several epitopes on caspase-3, one could expect these "doublets", as there would be a band present for the inactive protein, the active protein and a cleaved fragment. However, as caspases are known to be synthesized as enzymatically inert zymogens (12), one could expect a higher molecular weight band for the inactive C3L to be present in the earlier sampled cultures. A possible explanation for this discrepancy could be that the bacteria express this non-activated, caspase zymogen only when the cell density has reached a set level. In this model, when bacterial density increases, the bacteria would recognize the change in the environment and in turn would express a new subset of proteins, including the inactivated C3L protease. The expression of a pro-apoptotic factor in such an environment could be beneficial to the bacterial population, as having some portion of the population dying off could increase the survival of the overall population. Therefore, the doublet would be seen only at the 24 hour time point. There is precedent for such a hypothesis as several groups have shown that sufficient bacterial density as well as quorum sensing are necessary for bacterial programmed cell death and theoretically, the production of a C3L protein (5, 26). Separately, while there are doublets present in Figure 2, there are also other bands present. This does raise some questions regarding the specificity of the polyclonal antibody against caspase-3 alone (Fig. 2). It is possible that these additional bands may have arisen due to the nature of the polyclonal antibody, which could possibly bind to conserved enzymatic structures present on several proteases, or other proteins. If this were true, these results would suggest that there is structural homology between human and prokaryotic proteases at a conserved motif.

Of the 14 strains tested in the colourimetric assay, only the *S. maltophilia* H361-V and *P. aeruginosa* H823-S samples showed C3L activity. Interestingly, while these two strains also showed some of the strongest hybridization in the western blots, they did not have any unique bands present which differentiated them from the other positive

bacterial cultures. It is possible, that as the enzymatic assay was not optimized, only those samples with the greatest amount of C3L protein were able to cleave the substrate. However, the results of the colourimetric assay do not appear to be due to C3L concentration alone, as those samples which had strong banding present in the western blot did not have any results in the colourimetric assay (*P. aeruginosa* H822-S, H822-V), while samples which did not have any C3L protein present in the western blot showed low levels of substrate cleavage (*P. aeruginosa* H829-S, *S. maltophilia* H361-S). Alternatively, one could suggest that the cleavage of the substrate did not occur by the C3L protein, but instead may have occurred by a separate protease. This hypothesis is consistent with the broad range of the colourimetric substrate which can be cleaved by several eukaryotic proteases including caspase-3, -6, -7, -8, and -10. Given the sheer diversity of proteases present in prokaryotic cells and the number of proteins bound by the polyclonal α-caspase-3 antibody that have structural similarities to caspase-3 (Fig. 2), it is possible that the bacteria may have a non-caspase protease which could cleave the substrate. However, without further studies testing for other conventional apoptotic markers, it is difficult to conclude whether these putative C3L proteins are indeed involved in programmed cell death, or whether this enzymatic activity arose from an alternative protease.

One obvious question that arises from this set of observations, is that while the bacteria appear to be producing a C3L protease (Fig. 2) which can cleave a caspase-3 substrate (Fig. 3), the bacteria themselves do not appear to be decreasing in number. In fact the 48 hour sample of *S. maltophilia* H361-V showed a significantly larger pellet than the 24 hour sample, indicating continued bacterial growth over this period of time (data not shown). This result contrasts the work published by Gautam *et al.* (10), where they reported that the number of *X. campestris* AM2 grown in LB media decreased over time while the expression of C3L increased over a similar period (10). Our data would suggest that these results may have arisen due to coincidental effects, and in fact the presence and activity of this C3L protein do not determine cell death. However, as these experiments are simply initial results, it is necessary to continue further studies before one could reach such a conclusion.

FUTURE EXPERIMENTS

Due to the preliminary nature of this work, further experimentation should be performed in order to increase our understanding of the C3L protein that was expressed in *S. maltophilia, P. aeruginosa*, and *B. subtilis*. Although our data indicates the expression (*S. maltophilia* H361-V, *P. aeruginosa* H823-V and *P. aeruginosa* H822-V) and the activity (*S. maltophilia* H361 and *P. aeruginosa* H829) of a C3L protein, the expected positive correlation with a decrease in cell number was not seen.

Additional experiments to test for standard apoptotic markers (eukaryotic), such as the presence of nicked DNA and Annexin-V staining of phosphatidylserine in cell membranes, should be performed. The TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay detects for nicked DNA that is a characteristic marker of programmed cell death (10). The presence of fragmented DNA could also be detected via 2D agarose gel electrophoresis. Cells undergoing programmed cell death also show an increase of phosphatidylserine (PS) in the outer lipid monolayer of cell membranes. Annexin-V is a fluorescent dye that has a high affinity for PS and can be used to assay for its presence during the early stages of PCD (20). Previous experiments by Gautam *et al.* (10) utilized both the TUNEL assay and Annexin-V staining in *Xanthomonas* strains, indicating the competency of these assays for prokaryotes. A time-course of bacterial growth is recommended for both of these detection assays, as DNA fragmentation occurs at the later stages of apoptosis and accumulation of phosphatidylserine takes place at the earliest stage of apoptosis. Furthermore, comparing growth rates of the bacteria would allow one to both express the relationship between the C3L protein and programmed cell death and quantitate the relative level of expression and relative enzyme activity of the C3L protein.

Separately, it would be interesting to determine the sequence of the proteins to which the human anti-caspase-3 antibody binds. Two possible approaches for such an experiment could include doing an immunoprecipitation of the bacterial cell lysate and then sequence the resulting protein. Alternatively, it would be possible to simply extract the protein from a 2D acrylamide gel following a western blot and sequence the proteins present in the band by mass spectrophotometry.

Finally, due to technical difficulties experienced during the growth phase of this experiment, the standard bacterial cultures were grown at 26°C instead of the intended optimal 37°C. The experiment should be repeated at 37°C to ensure the causal factor in the expression and activity of C3L is strictly sub-optimal growth media.

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REFERENCES

1. **Aizenman, E., H. Engelberg-Kulka, and G. Glaser.** 1996. An *Escherichia coli* chromosomal "addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: a model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA **93:**6059-6063.
2. **Bast, R. C., Donald W. Kufe, Rapheal E. Pollock, Ralph R. Weichselbaum, James F. Holland, Emil Frei.** 2000. Cancer Medicine, 5th ed. Decker Inc., Hamilton, BC.
3. **Brooun, A., S. Liu, and K. Lewis.** 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents. Chemother. **44:**640-6.
4. **Cooper, G. M.** 2000. The Cell - A Molecular Approach, 2nd ed. Sinauer Associates Inc., Sunderland, MA.
5. **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:**6481-9.
6. **Davey, M. E., and A. O'Toole G.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847-67.
7. **Di Bonaventura, G., I. Spedicato, D. D'Antonio, I. Robuffo, and R. Piccolomini.** 2004. Biofilm formation by *Stenotrophomonas maltophilia*: modulation by quinolones, trimethoprim-sulfamethoxazole, and ceftazidime. Antimicrob. Agents. Chemother. **48:**151-60.
8. **Dow, J. M., L. Crossman, K. Findlay, Y. Q. He, J. X. Feng, and J. L. Tang.** 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. Proc. Natl. Acad. Sci. USA **100:**10995-11000.
9. **Drenkard, E.** 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microb. Infect. **5:**1213-9.
10. **Gautam, S., and A. Sharma.** 2002. Involvement of caspase-3-like protein in rapid cell death of *Xanthomonas*. Mol. Microbiol. **44:**393-401.
11. **Gerdes, K., P. B. Rasmussen, and S. Molin.** 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. U S A **83:**3116-20.
12. **Hengartner, M. O.** 2000. The biochemistry of apoptosis. Nature **407:**770-6.
13. **Kim, H. S., S. Y. Jeong, J. H. Lee, B. E. Kim, J. W. Kim, S. W. Jeong, and I. K. Kim.** 2000. Induction of apoptosis in human leukemia cells by 3-deazaadenosine is mediated by caspase-3-like activity. Exp. Mol. Med. **32:**197-203.
14. **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. Williams.** 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science **278:**294-8.
15. **Marissen, W. E., and R. E. Lloyd.** 1998. Eukaryotic translation initiation factor 4G is targeted for proteolytic cleavage by caspase 3 during inhibition of translation in apoptotic cells. Mol. Cell. Biol. **18:**7565-74.
16. **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:**2029-38.
17. **Naito, Y., T. Naito, and I. Kobayashi.** 1998. Selfish restriction modification genes: resistance of a resident R/M plasmid to displacement by an incompatible plasmid mediated by host killing. Biol. Chem. **379:**429-36.
18. **Nakayama, Y., and I. Kobayashi.** 1998. Restriction-modification gene complexes as selfish gene entities: roles of a regulatory system in their establishment, maintenance, and apoptotic mutual exclusion. Proc. Natl. Acad. Sci. U S A **95:**6442-7.
19. **Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, Munday, N. A., Raju, S. M., Smulson, M. E., Ting-Ting, Y., Violeta, L. Y., Miller, D. K.** 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature **376:**37-43.
20. **Ning, S. B., Y. C. Song, and P. Damme Pv.** 2002. Characterization of the early stages of programmed cell death in maize root cells by using comet assay and the combination of cell electrophoresis with annexin binding. Electrophoresis **23:**2096-102.
21. **Palleroni, N. J., and J. F. Bradbury.** 1993. *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al. 1983. Int. J. Syst. Bacteriol. **43:**606-9.
22. **Rice, K. C., and K. W. Bayles.** 2003. Death's toolbox: examining the molecular components of bacterial programmed cell death. Mol. Microbiol. **50:**729-38.
23. **Snyder, L.** 1995. Phage-exclusion enzymes: a bonanza of biochemical and cell biology reagents? Mol. Microbiol. **15:**415-20.
24. **Speert, D. P., J. E. Dimmick, G. B. Pier, J. M. Saunders, R. E. Hancock, and N. Kelly.** 1987. An immunohistological evaluation of *Pseudomonas aeruginosa* pulmonary infection in two patients with cystic fibrosis. Pediatr. Res. **22:**743-7.
25. **Thornberry, N. A., and Y. Lazebnik.** 1998. Caspases: enemies within. Science **281:**1312-6.
26. **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:**4585-92.
27. **Yarmolinsky, M. B.** 1995. Programmed cell death in bacterial populations. Science **267:**836-7.
28. **Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz.** 1993. The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell **75:**641-52.