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# Biofilm Development and Cell Death in the Marine Bacterium *Pseudoalteromonas tunicata*

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**The newly described green-pigmented bacterium *Pseudoalteromonas tunicata* (D2) produces target-specific inhibitory compounds against bacteria, algae, fungi, and invertebrate larvae and is frequently found in association with living surfaces in the marine environment. As part of our studies on the ecology of *P. tunicata* and its interaction with marine surfaces, we examined the ability of *P. tunicata* to form biofilms under continuous culture conditions within the laboratory. *P. tunicata* biofilms exhibited a characteristic architecture consisting of differentiated microcolonies surrounded by water channels. Remarkably, we observed a repeatable pattern of cell death during biofilm development of *P. tunicata*, similar to that recently reported for biofilms of *Pseudomonas aeruginosa* (J. S. Webb et al., J. Bacteriol. 185:4585–4595, 2003). Killing and lysis occurred inside microcolonies, apparently resulting in the formation of voids within these structures. A subpopulation of viable cells was always observed within the regions of killing in the biofilm. Moreover, extensive killing in mature biofilms appeared to result in detachment of the biofilm from the substratum. A novel 190-kDa autotoxic protein produced by *P. tunicata*, designated AlpP, was found to be involved in this biofilm killing and detachment. A Δ*alpP* mutant derivative of *P. tunicata* was generated, and this mutant did not show cell death during biofilm development. We propose that AlpP-mediated cell death plays an important role in the multicellular biofilm development of *P. tunicata* and subsequent dispersal of surviving cells within the marine environment.**

The newly designated genus *Pseudoalteromonas* resulted from the division of the genus *Alteromonas* into the two genera *Alteromonas* and *Pseudoalteromonas*, based on phylogenetic comparisons by Gauthier et al. (14). *Pseudoalteromonas* spp. have been isolated from diverse marine habitats globally (13, 18, 36, 49) and are frequently found in association with the surfaces of eukaryotic hosts. Species have been isolated from various animals, such as mussels (23, 25), puffer fish (47), tunicates (20), and sponges (26), as well as from a range of marine plants (13, 24, 56).

*Pseudoalteromonas* spp. are also known to produce a variety of extracellular compounds which inhibit or control adaptive and behavioral responses in many target organisms (19–21, 34). For example, the dark-green pigmented species *Pseudoalteromonas tunicata*, originally isolated from the tunicate *Ciona intestinales* (20), produces at least six novel extracellular compounds, each with inhibitory activity against a specific group of marine fouling organisms, including bacteria, invertebrate larvae, algal spores, diatoms, heterotrophic flagellates, and fungi (10–13, 19–21, 28). These inhibitory compounds are hypothesized to provide an advantage to *P. tunicata* during the competitive colonization of living marine surfaces (10, 11, 19–21).

One of the inhibitory compounds produced by *P. tunicata* is a novel 190-kDa antibacterial protein (AlpP) (28). AlpP inhibits the growth of both gram-positive and gram-negative bacteria, including terrestrial, medical, and marine isolates. Interestingly, logarithmic-phase growing cells of *P. tunicata* were found to be among the most sensitive to AlpP of the broad range of organisms tested, although stationary-phase cells become resistant (28). The role of this autocidal activity in *P. tunicata* is unclear. However, there are examples where autolysis plays an important in bacterial developmental processes (35). For example, in *Myxococcus xanthus*, a number of autocidal compounds are responsible for killing 80 to 90% of the cell population before fruiting body formation can occur (44, 55). More recently, a cell-cell signaling-mediated genetic mechanism was characterized whereby a subpopulation of *Bacillus subtilis* cells delay sporulation by killing sister cells and feeding on the nutrients that are released (15). Thus, autolysis, which appears undesirable to a single-cell organism, may be advantageous to a bacterial population at the multicellular level. In this study, we hypothesized that the autotoxic protein AlpP produced by *P. tunicata* may play a role in multicellular biofilm development.

Bacteria in biofilms are physiologically and morphologically different from their planktonic growing counterparts (3). In laboratory systems, biofilm bacteria often form highly differentiated three-dimensional structures (microcolonies) which become surrounded by a network of water channels. Microcolony formation has been demonstrated for most model biofilm-forming bacteria, e.g., *Escherichia coli*, *Vibrio cholerae*, and *Pseudomonas aeruginosa* (6, 8, 33, 52). Mature microcolo-

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nies can undergo complex differentiation. Dispersal of bacteria from the interior regions of microcolonies has been observed, apparently resulting in the formation of transparent voids inside the microcolonies (29, 45, 51). Furthermore, it was recently shown that killing and lysis occur reproducibly in localized regions in wild-type *P. aeruginosa* biofilms, inside microcolonies, by a mechanism that involves a genomic prophage of *P. aeruginosa* (54). It was proposed that cell death plays an important role in subsequent biofilm differentiation and dispersal, as surviving cells may benefit from the nutrients released during bacterial lysis (54). However, other roles for the death of a subpopulation of biofilm cells may be envisaged. For example, *P. tunicata* is thought to defend eukaryotic surfaces against further colonization and biofouling via production of its extracellular inhibitory compounds (20, 21). In this situation, autoregulation of biofilm formation by *P. tunicata* may be required to prevent detrimental overgrowth on the host surface.

In this study, we report that *P. tunicata* undergoes a highly reproducible pattern of cell death during normal development in a biofilm. We also demonstrate the involvement of a novel autotoxic protein, AlpP, in the killing process through the identification, sequencing, and subsequent site-directed mutagenesis of the corresponding gene *alpP*. We propose that AlpP-mediated killing may benefit a subpopulation of surviving cells and has an important role in subsequent biofilm development and dispersal in *P. tunicata*.

## MATERIALS AND METHODS

**Bacterial strains and media.** *P. tunicata* was routinely cultivated at room temperature in Väätänen nine-salt solution (VNSS) (37). The *P. tunicata alpP* mutant was maintained on VNSS medium containing the antibiotics streptomycin (100 μg $ml^{-1}$) and kanamycin (50 μg $ml^{-1}$). Biofilms were grown in marine minimal medium (42) containing 0.01% trehalose.

**Biofilm experiments.** *P. tunicata* wild-type and Δ*alpP* mutant strains were grown in continuous culture flow cells (channel dimensions, 1 by 4 by 40 mm) at room temperature as previously described (40). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately $10^9$ cells $ml^{-1}$ and incubated without flow for 1 h at room temperature. Flow was then started with a mean flow velocity in the flow cells of 0.2 mm $s^{-1}$, corresponding to laminar flow with a Reynolds number of 0.02. To investigate cell death during biofilm development, biofilms were stained with the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes Inc., Eugene, Oreg.). The two stock solutions of the stain (SYTO 9 and propidium iodide) were diluted to 3 μl $ml^{-1}$ in biofilm medium and injected into the flow channels. Live SYTO 9-stained cells and dead propidium iodide-stained cells were visualized with a confocal laser scanning microscope (CLSM) (Olympus) with fluorescein isothiocyanate and tetramethyl rhodamine isocyanate optical filters, respectively.

To provide statistically based, quantitative measurements during biofilm development, we characterized biofilm morphology with the COMSTAT program (17). Biofilms were stained with acridine orange (ProSciTech, Kelso, Australia), and 5 image stacks were recorded for 3 biofilms, resulting in 15 image stacks per time point. Images were acquired at 2-μm intervals through the biofilm at random positions in the flow cell at 3 time points (24, 72, and 120 h) as previously described (16) with the CLSM. The following parameters were assessed: maximum thickness (μm), average thickness (μm), total biomass ($\mu m^3\ \mu m^{-2}$), roughness coefficient, and surface-to-volume ratio ($\mu m^2\ \mu m^{-3}$).

**Identification and sequencing of *alpP* in *P. tunicata*.** Purification and characterization of the AlpP protein has previously been described (28). In the previous study, the multiple sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) subunits (60 and 80 kDa) of AlpP were found to share an identical N-terminal sequence for the first 27 amino acids. This sequence was used to design the following oligonucleotide probe (P1) based on codons most frequently used by the closely related genus *Vibrio* (41): 5′-ATG AAT CTG AAA ATT CAT CCA TCT GTT GGT GTT GCA CGT CTG GGT AAT TCT GAA ATG GAG GAT AAA ATT CTG TCT CGT GA-3′. The P1 probe was used to screen a *P. tunicata* genomic library constructed by using the phagemid vector λ Zap Express (Stratagene) and the enhanced chemiluminescence 3′ oligolabeling and detection kit (Amersham). The phagemid vector (pBK-CMV; Stratagene) was then excised from the phage into *E. coli* strain XLOLR (Stratagene) according to manufacturer's instructions. *P. tunicata* genomic DNA fragments extracted from the genomic library with probe P1 were sequenced by primer walking with a BigDye terminator cycle sequencing reaction mix (Applied Biosystems) and analyzed on an ABI 377 DNA sequencing system. To obtain sequence information further downstream in the antibacterial protein gene, the same genomic library was subsequently screened with a second oligonucleotide probe (P2, 5′-GAT CCG GTT TTC TAA AGT AAT CAT AAT AAG CCT GTC TTT TGC-3′) which targeted a region of the gene 994 bp downstream from the site corresponding to the N terminus of AlpP.

To determine whether both subunits of the AlpP protein originate from a single copy of the *alpP* open reading frame, we designed the oligonucleotide probe P3 (5′-ATC CAT CCC TCA GTC GGT GTT GCC-3′) by using the sequence obtained for the N-terminal region shared by the two subunits. *P. tunicata* genomic DNA digests were prepared by using a range of restriction enzymes (PstI, KpnI, EcoRI, BamHI, HaeII, ScaI, HindIII, and XbaI), resolved on a 0.75% agarose gel and transferred via Southern blotting to a polyvinylidene difluoride membrane (Bio-Rad) prior to probing.

**Site-directed mutagenesis of *alpP*.** To investigate the influence of AlpP on biofilm development, a Δ*alpP* mutant derivative of *P. tunicata* was generated by allelic displacement. The *alpP* gene (2.3 kb) was amplified with the following primers: forward, 5′-GAG AAT TCC ATA TGA ATT TAA AAA TCC ATC C-3′; reverse, 5′-AGT CTA AGC ATA TGG GAT CCT GCG TAA GTG ATA TCC C-3′. A kanamycin resistance ($Km^r$) cassette (1.2 kb) was amplified from the plasmid pUCR4K (Amersham) by using the following primers: forward, 5′-TAC TAG ATC TCA CGT GCG TCG ACC TGC AGG G-3′; reverse, 5′-GTG AAG ATC TCA CGT GCC GGA TCC GTC GAC C-3′. The *alpP* gene and $Km^r$ cassette were each cloned separately into pGEM T-Easy vectors according to the manufacture's instructions (Invitrogen). The $Km^r$ cassette was then inserted into *alpP* by using the PmlI restriction enzyme to create the plasmid pGEM *alpP*::$Km^r$.

The *alpP* knockout plasmid, pAP704, was constructed by inserting the *alpP*::$Km^r$ construct into the SmaI site of the suicide vector pGP704 (39), which was then transformed into *E. coli* SM10. The *P. tunicata* Δ*alpP* mutant was constructed by site-directed mutagenesis by conjugation with *P. tunicata* $Sm^r$ and *E. coli* SM10 containing the vector pAP704. Exconjugants with the *alpP*::$Km^r$ constructs inserted into the chromosome were selected by using VNSS plates supplemented with streptomycin (200 μg $ml^{-1}$) and kanamycin (85 μg $ml^{-1}$), and confirmation that the *alpP*::$Km^r$ cassette has been inserted into the genome of *P. tunicata* was obtained by PCR. Loss of autocidal activity in the concentrated supernatants of *P. tunicata* exconjugants was confirmed by using the drop plate assay for the detection of AlpP activity as previously described (28). Biofilm development of the *alpP* mutant was compared with that of wild-type *P. tunicata*. Flow cell experiments were carried out, and biofilms were stained with the LIVE/DEAD *Bac*Light bacterial viability kit as described above.

**Add-back of purified AlpP to *P. tunicata* Δ*alpP* mutant and wild-type biofilms.** To determine whether AlpP could restore killing in *P. tunicata* Δ*alpP* mutant biofilms, we added purified AlpP into flow cell channels containing *P. tunicata* Δ*alpP* mutant biofilms. We also added purified AlpP to young wild-type *P. tunicata* biofilms, before the normal onset of cell death, to determine whether AlpP could induce early killing in the wild-type strain. AlpP was prepared and purified from the supernatant as previously described (28). AlpP (10 to 12 μg in dialysis buffer [20 mM Tris, 0.3 M NaCl]) was injected into the flow cells with a syringe needle. Silicone tubing on either side of the flow cell was then blocked off by using tubing clamps. As a control, dialysis buffer was inoculated into separate flow cell channels. Biofilms were incubated at room temperature for 5 h without flow before staining with the LIVE/DEAD *Bac*Light bacterial viability kit and visualizing with the CLSM.

**TEM examination of AlpP.** Recently, cell death during development of *P. aeruginosa* biofilms was linked to the activity of a *P. aeruginosa* prophage (54). To investigate the possibility that AlpP is also a protein component of a bacteriophage or phage-like bacteriocin, we examined purified native AlpP by transmission electron microscopy (TEM). An AlpP-containing ion-exchange fraction was run on a native PAGE gel as previously described (28). The AlpP protein band was extracted from the gel (46) and eluted into 20 mM Tris (pH 7.4). Samples were placed onto a carbon- and Formvar-coated copper grid and fixed with 0.1% (vol/vol) glutaraldehyde in phosphate-buffered saline for 5 min. The grid was washed several times with phosphate-buffered saline (pH 7.0) and stained with 2% phosphotungstic acid for 30 s. The stained grid was examined on a Hitachi H700 TEM at an accelerating voltage of 75 kV.

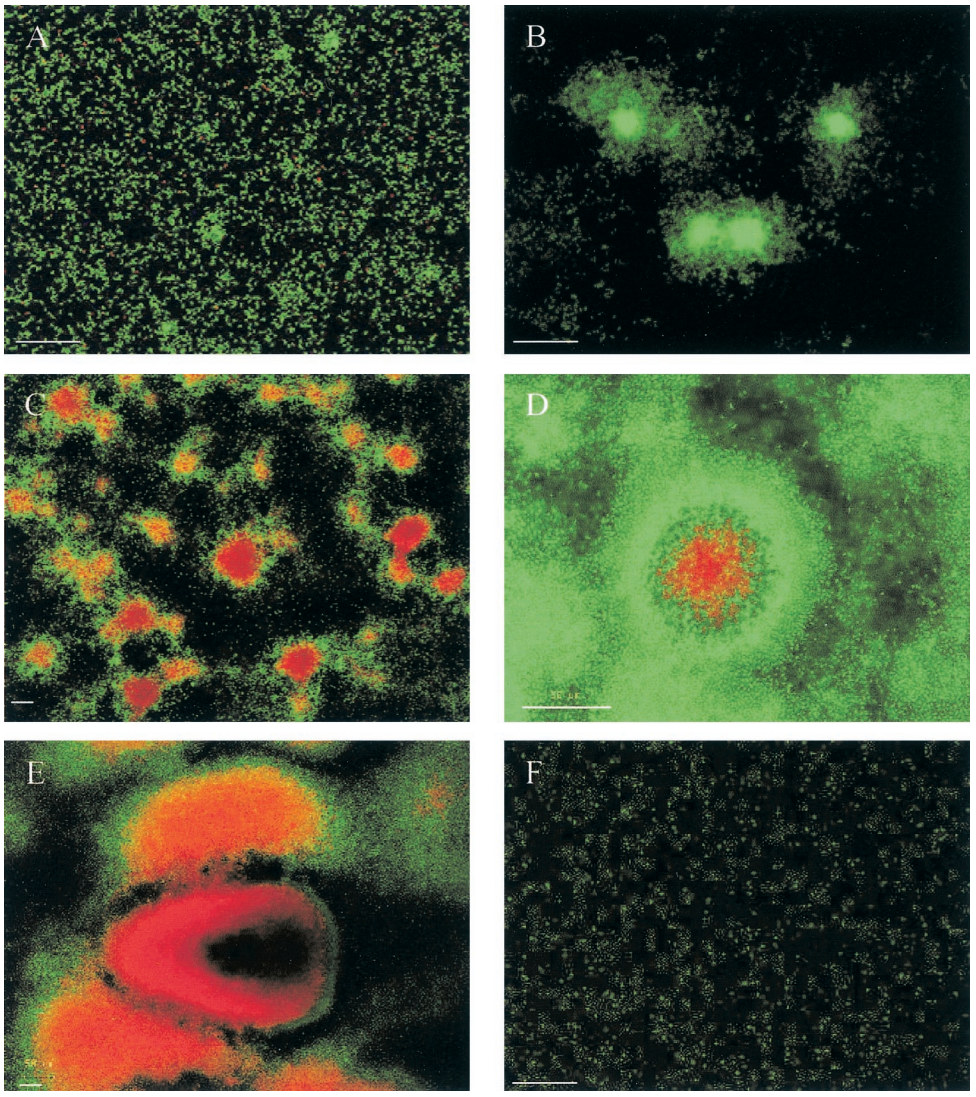


FIG. 1. Biofilm development and cell death of the *P. tunicata* wild-type strain. Biofilms were stained with the *Bac*Light LIVE/DEAD bacterial viability kit. Red propidium iodide-stained cells have a compromised cell membrane and are dead. Time points after inoculation are shown as follows: (A) 1 h; (B) 24 h; (C) 48 h; (D) 72 h; (E) 144 h; (F) 168 h. Bars, 50 μm.

**Nucleotide sequence accession number.** The primary nucleotide sequence data for the *alpP* gene has been deposited in the GenBank database under accession number AY 295768.

## RESULTS AND DISCUSSION

***P. tunicata* biofilm development and cell death.** We characterized *P. tunicata* wild-type biofilm development and cell death in glass flow cells by using the LIVE/DEAD *Bac*Light bacterial viability kit. Immediately after inoculation, single viable cells were observed attached to the substratum (Fig. 1A). After 24 h, microcolonies had developed (Fig. 1B) and no dead cells were visible within the biofilm. Between 48 and 96 h postinoculation, dead cells occurred in the interior portions of microcolonies and were surrounded by an outer layer of live cells (Fig. 1C and D). We observed dead cells and partially lysed cells as well as amorphous red propidium iodide-stained material, which was possibly DNA-containing debris from lysed cells. A subpopulation of cells in the region of killing remained viable. At this stage of biofilm development, the substratum was completely covered by bacteria. After 96 h, when killing had occurred inside all microcolonies, open voids within the regions of killing inside the microcolonies were observed (Fig. 1E). These open voids were similar to the voids and hollow microcolonies recently described for *P. aeruginosa* (45, 51). Regions of extensive killing subsequently occurred within and around microcolonies, and the biofilm structure started to disrupt and detach. Once the biofilm had dispersed, no microcolonies could be observed while only single live cells remained attached within the flow cell (Fig. 1F). Similar results were observed in five additional sets of experiments.

Comparable cell death within mature microcolonies has recently been demonstrated in the opportunistic human pathogen *P. aeruginosa* (54), and other observations have shown that loss of viability also occurs inside microcolonies in biofilms

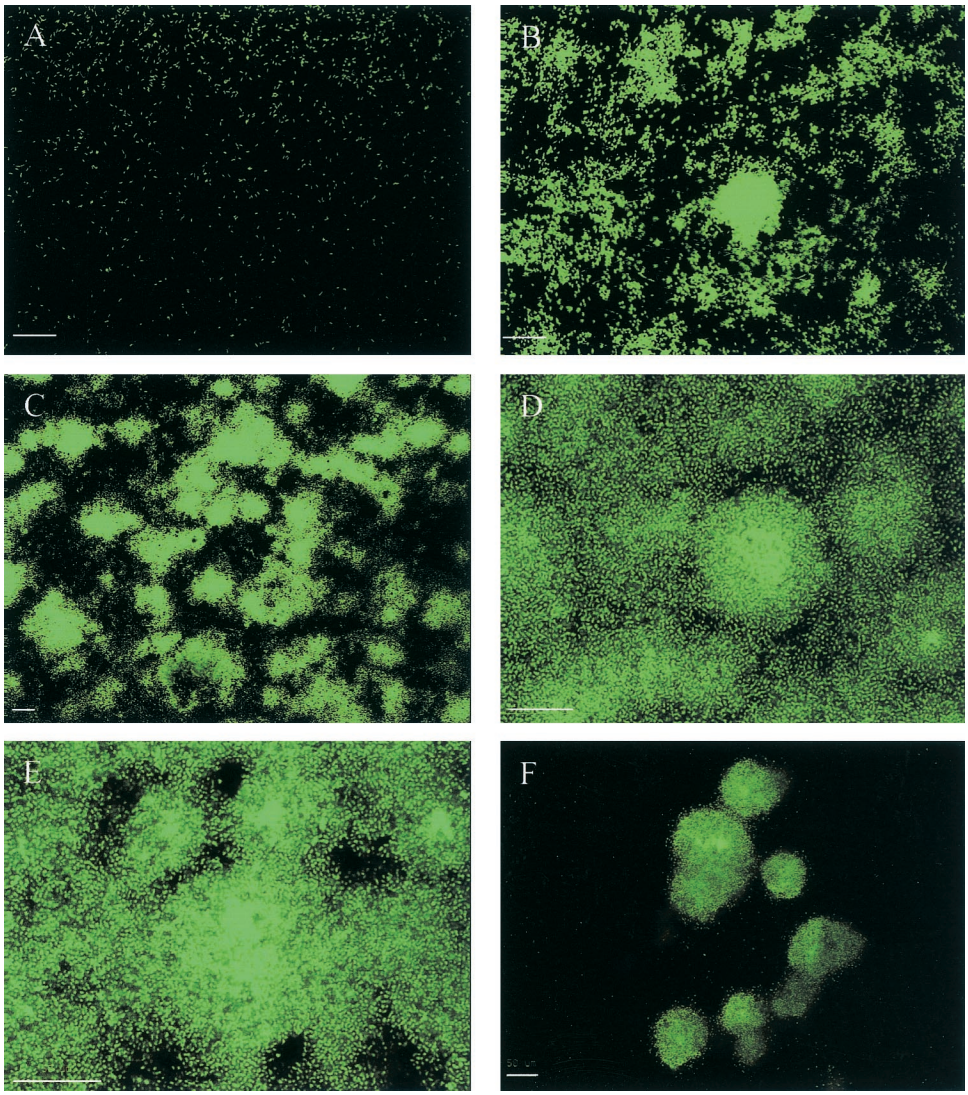


FIG. 2. The *P. tunicata* AlpP mutant does not show cell death during biofilm development. Biofilms were stained with the LIVE/DEAD *Bac*Light bacterial viability kit. Time points after inoculation are shown as follows: (A) 1 h; (B) 24 h; (C) 48 h; (D) 72 h; (E) 144 h; (F) 168 h. Bars, 50 μm.

formed by oral bacteria (2, 22). Thus, cell death inside microcolonies may be widespread among biofilm-forming bacteria, and we hypothesize that these killing events play an important role in biofilm development and dispersal. For example, nutrients released during cell death may benefit other bacteria within the biofilm because they provide an energy source for continued development and dispersal. However, little is known about the mechanism(s) by which hollow microcolonies are formed or by which cells disperse from the internal regions inside microcolonies. Generally, large-scale biofilm detachment and sloughing is known to be influenced by both physical and physiological properties. Hydrodynamic conditions such as shear stress and velocity of fluids can greatly affect the biofilm matrix and detachment of biofilms (5, 43). In addition to hydrodynamics, biofilm detachment is also influenced by environmental sensors and nutritional conditions. For example, it was shown that the global regulatory protein CsrA (carbon storage regulator) serves as an activator of biofilm dispersal through the regulation of intracellular glycogen biosynthesis and catabolism in *E. coli* (27). In the present study, we investigated whether the autotoxic protein AlpP of *P. tunicata* plays a role in biofilm killing and dispersal.

**Identification and sequencing of *alpP*.** *P. tunicata* was previously found to produce a multisubunit (60 and 80 kDa) protein with autotoxic activity (28), which it releases into its surrounding environment when grown both as colonies on plates and in liquid culture (28). The complete sequence of the gene for the *P. tunicata* autotoxic protein was determined from DNA fragments drawn from a genomic library of this bacterium with the probes P1 and P2. The open reading frame of this gene encodes a 748-residue protein with a calculated molecular mass of 80.9 kDa, in close agreement with the large 80-kDa subunit observed in sodium dodecyl sulfate-PAGE. The gene was designated *alpP* (for "autolytic protein,

*Pseudoalteromonas*"), and the primary nucleotide sequence data for the *alpP* gene has been deposited in the GenBank database (see above).

Because both the 80- and 60-kDa subunits of AlpP share an identical N-terminal sequence (28), we examined whether these subunits originate from the same gene. Southern analysis of *P. tunicata* genomic digests was carried out with the DNA probe P3 (specific for the sequence of the shared N-terminal region). The P3 probe hybridized to a single band in all digests (data not shown), indicating that both the 60- and 80-kDa protein subunits originate from a single gene. The mechanism by which the 60-kDa subunit is expressed from *alpP* is not clear; however, possibilities include the posttranslational proteolytic cleavage of the larger 80-kDa subunit or early termination of transcription of *alpP*.

The sequence obtained contains the elements necessary for transcription, with promoter prediction analysis revealing a putative promoter region upstream of the start codon and a ribosome-binding site, 5′-AGGA-3′, preceding the initiation codon. The AT content is also high (70%) where strand-opening is predicted to occur, from the −10 region through to the first three codons of the open reading frame. The original publication of the first 18 N-terminal amino acids of AlpP indicated that it was a previously undiscovered protein (28). The entire predicted amino acid sequence of AlpP presented here shows identity with only three hypothetical proteins, which were more-recently entered into GenBank. These matches were found within the genomes of *Chromobacterium violaceum* ATCC 12472 (GenBank accession number NP_902938), *Magnetococcus* sp. strain MC-1 (GenBank accession number ZP_00044814), and *Caulobacter crescentus* CB15 (GenBank accession number NP_419374), with identities of 35, 32, and 27%, respectively, each being hypothetical proteins of no known function.

Previously, killing in *P. aeruginosa* biofilms has been linked to the activity of a prophage that is encoded within the genome of *P. aeruginosa* (54). We therefore examined native PAGE-purified AlpP by using TEM to investigate whether AlpP may be a component of a bacteriophage or bacteriophage-like bacteriocin. However, images obtained from the electron microscope clearly showed no phage or phage-like particles in the purified AlpP band (data not shown). Although investigations by Kivelä et al. (30, 31) have shown that phage are present in at least two *Pseudoalteromonas* species, our results indicate that cell death in *P. tunicata* biofilms does not involve bacteriophage. *P. tunicata* and *P. aeruginosa* therefore appear to have acquired different mechanisms that cause cell death during biofilm development.

**Biofilm development of a *P. tunicata alpP* mutant.** To investigate the role of *alpP* during *P. tunicata* biofilm growth, a Δ*alpP* mutant derivative of *P. tunicata* was generated. Biofilm development of the Δ*alpP* strain was investigated in comparison to wild-type *P. tunicata*. In a similar fashion to the wild-type, the *alpP* mutant formed a differentiated biofilm with a three-dimensional structure consisting of microcolonies (Fig. 2). We also characterized biofilms of the wild-type and the Δ*alpP* mutant strain with the biofilm quantification software COMSTAT (17). The results are summarized in Table 1. All assessed parameters showed no significant differences between the wild-type and the mutant strain after 1, 3, and 5 days of biofilm development. Thus, our data show that the *alpP* mutant is still capable of forming the normal biofilm architecture characteristic of wild-type *P. tunicata*.

TABLE 1. Analysis of *P. tunicata* wild-type and Δ*alpP* biofilms with COMSTAT

| Parameter (units) | Time (h) | Result for: | |
|---|---|---|---|
| | | Wild type | Δ*alpP* |
| Maximum thickness (μm) | 24 | 10.57 ± 3.21 | 16.67 ± 4.68 |
| | 72 | 28.29 ± 5.59 | 24.00 ± 4.62 |
| | 120 | 38.00 ± 5.29 | 37.60 ± 2.19 |
| Avg thickness (μm) | 24 | 2.01 ± 0.48 | 2.15 ± 1.98 |
| | 72 | 14.45 ± 1.96 | 10.63 ± 3.62 |
| | 120 | 31.64 ± 6.7 | 24.95 ± 1.44 |
| Total biomass ($\mu m^3\ \mu m^{-2}$) | 24 | 2.12 ± 0.46 | 2.00 ± 1.78 |
| | 72 | 11.77 ± 1.41 | 10.49 ± 4.87 |
| | 120 | 24.79 ± 5.64 | 27.38 ± 1.35 |
| Roughness coefficient | 24 | 1.08 ± 0.21 | 1.38 ± 0.48 |
| | 72 | 0.25 ± 0.07 | 0.49 ± 0.36 |
| | 120 | 0.11 ± 0.04 | 0.14 ± 0.02 |
| Surface-to-biovolume ratio ($\mu m^2\ \mu m^{-3}$) | 24 | 3.56 ± 0.46 | 2.12 ± 0.36 |
| | 72 | 2.80 ± 0.58 | 1.36 ± 0.43 |
| | 120 | 2.43 ± 0.66 | 0.52 ± 0.09 |

However, unlike wild-type *P. tunicata*, the *alpP* mutant did not undergo cell death at any stage of biofilm development, and no hollow cavities within microcolonies were observed. Moreover, detachment of the biofilm was considerably delayed, with the majority of microcolonies persisting after 9 days (wild-type biofilms were completely dispersed after 7 days) (Fig. 2F). These results suggest that AlpP-mediated killing affects both dispersal from within the interior portions of attached microcolonies as well as large-scale detachment of the whole biofilm.

**Add-back of purified AlpP to *P. tunicata* biofilms.** To provide direct evidence that AlpP is involved in cell death and subsequent detachment in *P. tunicata* biofilms, we added purified AlpP to *P. tunicata* Δ*alpP* 48-h biofilms in an attempt to restore AlpP-mediated killing in the biofilm. Similar to wild-type *P. tunicata*, the autotoxic protein caused cell death in the centers of microcolonies and also killed other cells throughout the biofilm (Fig. 3A). We also added AlpP protein to young wild-type biofilms (30 h) before the normal onset of killing within the microcolonies. Add-back of AlpP induced early killing in *P. tunicata* wild-type biofilms (Fig. 3C). Cell death occurred within microcolonies as well as in other regions of the biofilm. Interestingly, cells on the exterior of microcolonies were not killed by add-back of AlpP. While we understand that AlpP-mediated cell death is growth-phase-regulated in *P. tunicata* (28), the mode of action of AlpP is not fully understood. Thus, the means by which there is differential killing of subpopulations of cells within the biofilm remains to be fully explored.

**Proposed ecological and developmental roles of AlpP-mediated cell death.** In this study, the inhibitory marine bacterium *P. tunicata* demonstrated a repeatable pattern of cell death during normal biofilm development. The involvement of the autotoxic protein AlpP suggests that this process is an evolved capacity and may benefit the bacteria at the multicellular level

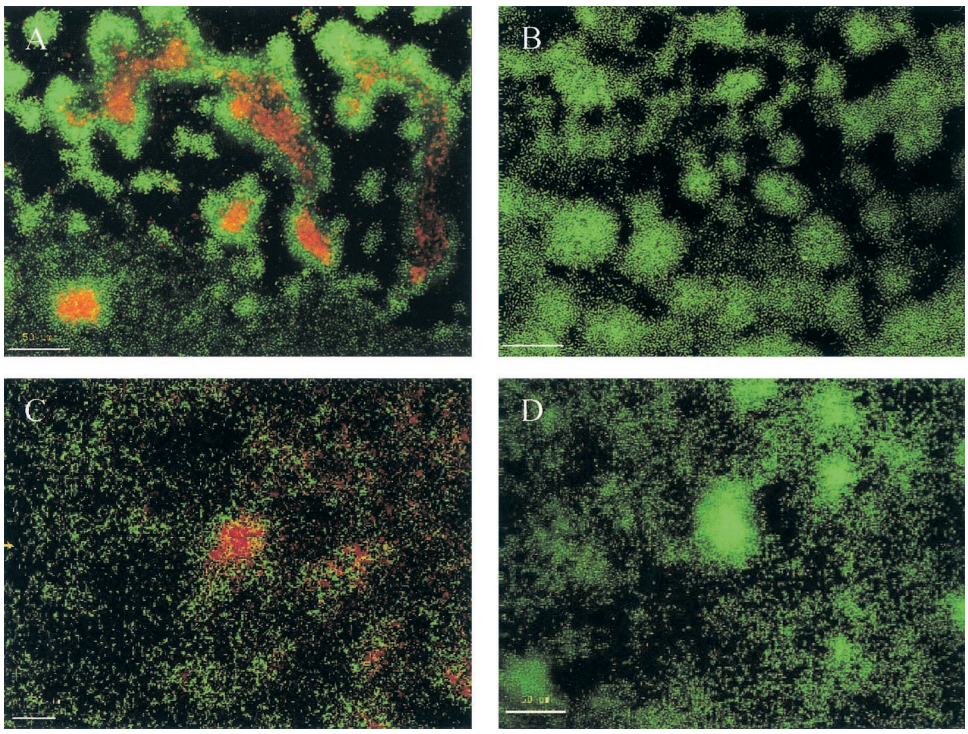


FIG. 3. Addition of purified of AlpP to *P. tunicata* biofilms. Biofilms were stained with the LIVE/DEAD *Bac*Light bacterial viability kit. (A) Add-back of AlpP to *P. tunicata* Δ*alpP* 48-h biofilms; (B) *P. tunicata* Δ*alpP* mutant biofilm plus buffer control (20 mM Tris, 0.3 M NaCl); (C) AlpP added back to young (30 h) *P. tunicata* wild-type biofilms before the normal onset of killing within the biofilm; (D) *P. tunicata* wild-type biofilm plus buffer control (20 mM Tris, 0.3 M NaCl). Bars, 50 μm. Similar results were obtained in four replicate experiments.

in a similar manner to that previously described for autolysis in *M. xanthus* and *B. subtilis* (15, 44, 55). Although the experiments presented here were carried out in an artificial flow cell environment, differentiated microcolony structures, similar to those reported here for *P. tunicata* biofilms, are commonly observed in natural and medical situations (1, 4, 48, 53). Moreover, several recent publications suggest that similar processes of cell death occur in microcolonies in more complex biofilms, such as those of dental plaque (2, 22) and within the biofilm flocs of wastewater treatment processes (38). Future experimentation will address the possible benefits that *P. tunicata* and other bacteria derive from processes of cell death during biofilm development.

One possibility is that cell death may function in *P. tunicata* biofilm dispersal. We observed that cell death occurred in localized regions within microcolonies of *P. tunicata* biofilms but that a subpopulation of cells remained viable in these regions. We propose that killing and lysis benefit this subpopulation of cells (through the release of nutrients and DNA) which then undergo continued differentiation and dispersal.

Another possible function of AlpP-mediated cell death in *P. tunicata* is that it may regulate biofilm growth on the surfaces of living hosts in the marine environment. Many sessile algae and animals have evolved defense mechanisms against fouling by producing metabolites that can influence the settlement, growth and survival of other organisms (for examples, see references 7 and 9). However, algae and animals lacking chemical and nonchemical defenses are thought to rely on secondary metabolites produced by associated surface bacteria, such as *P. tunicata*, as their defense against fouling (19, 32, 50). Cell death and dispersal of a subpopulation of cells within *P. tunicata* biofilms may protect its host against uncontrolled biofilm formation and fouling by *P. tunicata* itself.

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