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Purification and Characterization of a Novel Antibacterial Protein from the Marine Bacterium D2

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A biofilm-forming marine bacterium, D2, isolated from the surface of the tunicate *Ciona intestinalis*, was found to produce a novel, 190-kDa protein with antibacterial activity. The protein contained at least two subunits of 60 and 80 kDa, joined together by noncovalent bonds, and was shown to be released by D2 cells into the surrounding medium during stationary phase. N-terminal sequence analysis revealed no close similarity of this protein to any other proteins within the Swiss Prot database. Bacteriocidal activity against a wide variety of marine and medical bacterial isolates was observed, 77% of the strains tested being sensitive to the protein. Bacterial strains varied in their resistance to the D2 protein, with D2 itself being among the most sensitive with an MBC in liquid suspension of 4 μg/ml. An apparent increased resistance of D2 to the protein as the cells progressed further into stationary phase was observed and seen as a possible explanation for its survival despite the production of an autoinhibitory factor. The ability of the D2 bacterium to produce an antibacterial factor in addition to its inhibitory effects on marine invertebrates and algae (S. Egan et al., unpublished data) indicates that D2 has the potential to greatly affect the survival of a range of colonizers of the marine surface environment.

The marine surface environment is a site of intense competition for living space by a wide variety of organisms. Bacteria are generally recognized as primary colonizers of this habitat (1) and are able to rapidly form biofilms over freshly exposed surfaces. The prominence of bacteria during early colonization events and their almost universal presence on marine surfaces, including those of benthic marine invertebrates and algae (11, 16), would indicate that they have the potential to strongly influence the establishment of other colonizers in this environment.

An increasing number of compounds with antibacterial activity have recently been found to be produced by a variety of organisms present in the marine surface environment. The red alga *Delisea pulchra* has been shown to produce halogenated furanones with activity against representatives of the three major groups of fouling organisms, including invertebrate larvae, macroalgae, and bacteria (2). The antibacterial compounds produced by this alga were shown to be significantly more inhibitory than the antibiotic gentamicin. Four species of bryozoans isolated from Tasmanian coastal waters were shown to exhibit antibacterial activity (16). Bryozoan extracts were assayed for antibacterial effects upon 10 bacterial strains isolated from marine biofilms and appeared to be selective in their activity, each bryozoan species producing an extract which was most potent against a specific bacterial species.

A wide variety of benthic marine invertebrates appear able to influence their associated microbial flora through the production of antibacterial secondary metabolites. *Polysyncraton lacazei* (Giard), a colonial tunicate of the western Mediterranean, was shown to possess several secondary metabolites that together efficiently inhibit the growth and/or survival of all potential colonizers tested: bacteria, fungi, unicellular algae, and invertebrate larvae (14). Extracts from the tunicate were assayed against a collection of five bacterial strains consisting of three benthic bacteria isolated from the sediment surface in the immediate vicinity of the extracted tunicate population, one epibiotic strain (*Corynebacterium* sp.), and a laboratory strain of *Pseudomonas aeruginosa*. Moderate to strong bacteriostatic activity was detected against all five of these strains. Several fractions were shown to be responsible for this activity, the main ones being believed to contain mainly lipids and proteins. The sponge *Lissodendoryx isodictyalis* was also shown to produce metabolites with potent antifouling activity, which included antibacterial effects (12).

A marine bacterium, D2, was recently isolated from an adult tunicate by Holmström et al. (5) and shown to produce extracellular components able to kill the larvae of the marine invertebrates *Balanus amphitrite* and *Ciona intestinalis*. Recent studies have shown that, in addition to this activity, the D2 bacterium is able to release products into its surrounding environment which are inhibitory against several major groups of biofouling organisms including algae (3) and diatoms. The data presented in this paper further widen the spectrum of organisms known to be inhibited by products secreted by the D2 bacterium by adding bacteria to the list of target organisms. This is demonstrated with the discovery of a protein, produced by D2, which is inhibitory against a variety of bacterial species.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The marine bacterium D2 was originally isolated from an adult tunicate, *Ciona intestinalis*, collected from waters off the Swedish coast (5). The set of 20 unidentified marine strains used in the initial toxicity screening (growth medium concentration assay with a 50-kDa cutoff filter) was isolated from rock surfaces off the Swedish coast. *Proteus vulgaris*, *Proteus mirabilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Vibrio anguillarum*, *Bacillus subtilis*, *Bacillus rumilus*, *Bacillus globi*, and *Serratia liquefaciens* were taken from the culture collection at the University of New South Wales. The set of 24 unidentified marine surface bacterial isolates used in relative sensitivity experiments (including C3 and S7) was isolated from rock and marine algal surfaces off the coast of Sydney, Australia. All strains were stored at −70°C.

Cultures of D2, the set of 20 unidentified marine strains isolated in Sweden, and the 24 marine strains isolated in Australia were routinely grown and maintained in complex marine growth medium (VNSS) (9). *Proteus vulgaris*, *Proteus*

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mirabilis, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, and *Salmonella typhimurium* were maintained in Luria-Bertani broth (8) at 37°C whereas the remaining culture collection isolates were maintained with Luria-Bertani broth supplemented with 1% NaCl. All cultures were grown at 28°C with shaking unless otherwise stated.

Preparation of concentrated bacterial supernatant. The cells from a 24-h culture were harvested by centrifugation (9,000 × *g* for 20 min) and resuspended in artificial seawater (nine-salts solution) at a density of 0.6 g of bacterial cells per ml of NSS. This suspension was incubated under static conditions at 28°C for 24 h, after which the cells were removed by centrifugation (15,000 × *g* for 30 min). The supernatant was filter sterilized before further use.

Assays for antibacterial activity by inhibition zone formation. (i) Growth medium concentration (used for initial screenings). The growth medium from a 24-h D2 culture was concentrated with pressure filtration and a 50-kDa cutoff filter (Amicon). Both the concentrated medium and the used filter pieces were assayed for toxicity by placing them on VNSS agar plates just after the surface of the plate had been inoculated with target cells.

(ii) Overlay technique. Single D2 colonies were grown for 7 days. Target cells were grown to stationary phase and mixed with 0.6% VNSS agar at 45°C at a density of 0.4 ml of target culture per 3 ml of agar. Each 7-day culture plate was overlaid with 3 ml of this mixture and incubated overnight.

(iii) Drop test (assay for bacteriocidal activity). Concentrated D2 supernatant was prepared as described above. An overnight broth culture of the target strain was prepared, and 100 μl of the culture was spread over the surface of an agar plate of the growth medium appropriate to that strain. Drops containing 20 μl of D2 supernatant were placed on the target plates, which were then incubated overnight. Live cells could not be revived from the inhibition zones formed in sensitive strains when swabs were taken from this region and reinoculated onto fresh agar. No cells were recoverable from the inhibition zones of all assays taken as positive for bacteriocidal activity. Relative sensitivities of different strains to the bacteriocidal effect of the protein were estimated with serial dilutions.

Identification of an antibacterial protein band resolved by native gel electrophoresis. Samples with antibacterial activity were suspended in sucrose-dye solution (5% sucrose, 0.01% bromophenol blue) and resolved by polyacrylamide gel electrophoresis (PAGE) with an 8% gel. The gels were washed for 20 min in distilled water prior to being placed on VNSS agar plates, after which stationary-phase target cells were spread over the gel surfaces.

Antibacterial protein purification. The supernatant from high-density D2 suspensions was prepared as described for use in the drop test assay, after which ammonium sulfate was added to a concentration of 60%. The precipitated fraction was collected by centrifugation (15,000 × *g* for 30 min), resuspended in 20 mM Tris buffer (pH 7.5), and dialyzed against this buffer overnight (Selbys; 15,000 molecular weight cutoff). The sample was passed through a 5-ml Econo-Pac Q anion-exchange cartridge (strongly basic anion exchanger; Bio-Rad Laboratories) from which the bacteriocidal fraction was eluted at between 0.1 and 0.2 M NaCl at pH 7.5. The bacteriocidal fraction was identified at each step by the drop test. All protein assays were carried out with a bicinchoninic acid protein assay kit (Sigma).

The bacteriocidal fraction eluted from the ion-exchange column was dialyzed overnight against 0.001 M NaCl and passed through a 5-ml Econo-Pac hydroxyapatite cartridge (Bio-Rad), which was then washed with 1 M NaCl and 0.01 M phosphate buffer prior to elution of the protein with 0.4 M phosphate buffer. The purified fraction was stored at −20°C after freeze-drying.

Protein subunit structure. The following treatments were carried out upon samples of purified antibacterial protein to determine whether it contains subunits. The effects of treatments with β-mercaptoethanol and heat upon the subunit structure of the protein were visualized by sodium dodecyl sulfate (SDS)-PAGE. The purified protein was dissolved in SDS-PAGE treatment buffer (0.062 M Tris-Cl [pH 6.8], 4% SDS, 20% glycerol) and divided into four samples. The first sample was exposed to 5% β-mercaptoethanol, the second was heated in a boiling water bath for 90 s, and the third was exposed to both treatments. The fourth sample was left untreated as a control. The antibacterial protein was detected within these samples by SDS-PAGE on 8% 1.5-mm polyacrylamide gels stained with Coomassie brilliant blue (Bio-Rad).

N-terminal sequence analysis. The N-terminal amino acid sequence of both of the subunits of the antibacterial protein was determined. The purified protein fraction was treated with both 5% β-mercaptoethanol and heat as described above, and the bands were resolved by SDS-PAGE on 8% 1.5-mm polyacrylamide gels and electroblotted to Problott polyvinylidene difluoride membrane with a CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer system. The blotted proteins were stained on the membrane with amido black.

Heat treatment. Solutions of 20 μg of purified antibacterial protein per ml were exposed to 80°C heat for 10 min before exposure to D2 target cells by the drop test assay for bacteriocidal activity.

pH stability. Solutions of 20 μg of purified antibacterial protein per ml in nine-salts solution were adjusted to a range of pH values and incubated at 28°C for 1 h. The pH value of each solution was readjusted to its original pH, and its bacteriocidal activity was measured by the drop test assay.

Protein release during the growth curve of D2 cells. The release of antibacterial protein by D2 into its growth medium at different stages of the growth curve was determined as follows. One liter of VNSS growth medium was inoculated with 10 ml of precultured D2 cells and incubated as a shaking batch culture at 28°C. Cell density was measured throughout the growth curve. Samples of growth medium were taken at 4.5, 6.5, 7.5, 8.5, and 25 h and filter sterilized, and the antibacterial protein from these samples was concentrated onto an ion-exchange column as described in the purification protocol. The antibacterial protein was detected within these samples by SDS-PAGE on 8% 1.5-mm polyacrylamide gels stained with Coomassie brilliant blue.

MBCs and minimum growth inhibitory concentrations. The concentration of antibacterial protein necessary to result in cell death and to inhibit the growth of a variety of marine bacterial isolates in solution was determined as follows. Purified protein in sterile VNSS was added to the wells of a 96-well plate at a series of dilutions ranging from 0 to 800×. Bacterial cells were inoculated at time zero, and the growth rates were measured by absorbance. Cell viability was checked at 24 h by streaking samples onto VNSS agar plates.

RESULTS

Antibacterial activity of D2 supernatant. (i) Growth medium concentration. In order to search for antibacterial factors released by the D2 bacterium, growth medium from a 24-h D2 culture was concentrated onto a 50-kDa filter and the filter pieces were assayed against the set of 20 unidentified bacterial strains isolated from marine rock surfaces in Sweden. Unused growth medium was concentrated onto another 50-kDa filter which was used as control. Of the strains tested, 16 of 20 showed a clear zone indicating an absence of bacterial growth around the filter pieces, whereas no such zones were observed around the control filter pieces (data not shown). The <50-kDa fraction, concentrated by freeze-drying, produced no inhibition zones. This demonstrated that D2 cells in a 24-h batch culture release an antibacterial factor of >50 kDa into their surrounding medium.

(ii) Overlay technique. By the overlay technique, 14 of the 20 strains isolated from Sweden showed clear zones of nongrowth above and around 7-day D2 colonies on VNSS plates when placed in an overlay of nutrient agar (data not shown). This demonstrated that D2 cells in colonies on VNSS agar produce an antibacterial factor. The same strains that were sensitive in the overlay assay were the same ones found to be sensitive in the initial assay with filter pieces, which indicates that the same factor may be involved.

(iii) Drop test (for the detection of bacteriocidal activity). Supernatant from a high-density D2 culture was prepared and assayed against cells from a 24-h D2 culture by the drop test. Inhibition zones were observed, indicating that D2 produces a product which is bacteriocidal towards itself (data not shown). The concentrated D2 supernatant was also bacteriocidal against *V. anguillarum* cells.

In order to determine whether other strains grown at high density produce bacteriocidal products active against themselves, supernatant concentrates were prepared from high-density cultures of four of the marine bacterial isolates from Sweden, as well as *V. anguillarum* and the clinical isolates *Proteus vulgaris*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella typhimurium*. When assayed against the target organisms D2 and *Vibrio anguillarum*, by the drop test, none of the supernatants, except that from D2, produced inhibition zones in either of the target strains (data not shown). In addition, none of the strains, except D2, were shown to be autoinhibitory under these conditions.

Identification of an antibacterial protein band resolved by native gel electrophoresis. The major proteins present in concentrated, cell-free bacterial growth medium were visualized by native gel electrophoresis and Coomassie blue staining. A single band of antibacterial activity, which corresponded to a prominent protein band, was observed by the in-gel assay technique. The band of antibacterial activity and a corresponding Coomassie blue-stained gel of the purified antibacterial protein are shown in Fig. 1. These data, together with the size

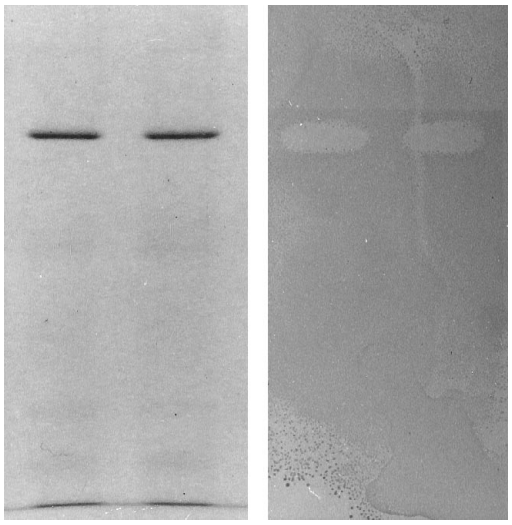

FIG. 1. In-gel toxicity assay for the antibacterial protein produced by D2. Antibacterial protein was purified from the medium of a high-density culture of D2 cells and run on an 8% polyacrylamide gel in the absence of SDS and β-mercaptoethanol. The gel was separated into two halves (each containing three lanes), one of which was stained for protein with Coomassie blue (left panel). The other half was washed for 20 min to remove residual acrylamide and placed on a VNSS agar plate, and its surface was inoculated with log-phase D2 cells. Zones of inhibition corresponding to the protein band were visualized the following day (right panel).

(>50 kDa) of the antibacterial factor and its loss of activity upon heating for 10 min at 80°C, were suggestive of a protein.

Antibacterial protein purification. The antibacterial protein was purified from concentrated D2 supernatant by ion-exchange and hydroxyapatite chromatography as described in Materials and Methods. Table 1 summarizes the purification. About 2 mg of purified protein was obtained from 150 ml of concentrated supernatant. The specific activity of the purified material was increased about 70-fold over that of the crude supernatant, and on SDS-PAGE (without prior treatment with β-mercaptoethanol and heat), it gave a single band of 190 kDa. For the purposes of this purification, one unit of antibacterial activity was defined as the minimal amount necessary to produce an inhibition zone in log-phase D2 target cells growing on the surface of an agar plate by the drop test.

Protein subunit structure. Samples of purified protein which had undergone treatment with 5% β-mercaptoethanol, heat only, or heat and β-mercaptoethanol and the untreated control were fractionated by SDS-PAGE to observe whether any of the treatments would split the protein into subunits. Protein which was treated with 5% β-mercaptoethanol only and the untreated control sample ran as a single band of approximately 190 kDa in size compared with molecular weight standards. This would suggest that the antibacterial protein does not contain subunits joined together by covalent bonds alone. Upon treatment with β-mercaptoethanol and heat, or heat only, the protein ran as two bands of 60 and 80 kDa in molecular mass although the relative intensity of the 80-kDa band is higher with the heat-only treatment (Fig. 2). This suggests that covalent interactions do contribute to the structure of the protein complex and that the 80-kDa band may represent more than one subunit. Clearly, this protein consists of at least two subunits; however, more information would be necessary to determine the exact size and structure of the subunits which make up the antibacterial protein. Neither the 60- nor the 80-kDa band produced inhibition zones when assayed within the gel for antibacterial activity as described above (data not shown), indicating that neither subunit is active in isolation.

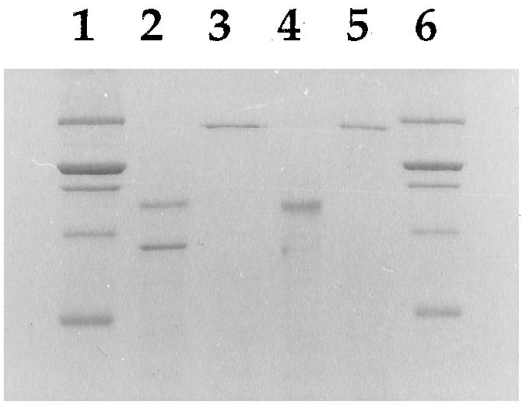

FIG. 2. An SDS–8% PAGE gel of the purified antibacterial protein after treatments aimed at breaking intersubunit bonds. Lanes 1 and 6, molecular weight standards. Lane 2, 10% β-mercaptoethanol and heat. Lane 3, 10% β-mercaptoethanol only. Lane 4, heat only. Lane 5, no treatment.

TABLE 1. Purification of the antibacterial protein from the medium of concentrated D2 cultures[a]

Step	Activity (U)	Protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Supernatant	500	103.5	4.83	1	100
NH_4SO_4 precipitation	425	29.3	14.5	3	85
Ion-exchange chromatography	225	2.3	97	20	45
Hydroxyapatite chromatography	224	0.7	345	71	45

[a] The protein was purified by ammonium sulfate precipitation at 60% followed by anion-exchange chromatography and hydroxyapatite chromatography. For the purposes of this purification, one unit of specific antibacterial activity was defined as the minimal amount necessary to produce an inhibition zone in D2 target cells growing on the surface of an agar plate by the drop test. All protein assays were carried out with a bicinchoninic acid protein assay kit (Sigma).

N-terminal sequence analysis. A sample of purified protein was prepared for N-terminal sequencing as follows. The sample was heated at 100°C for 90 s in the presence of 5% β-mercaptoethanol and resolved into two bands with an SDS–8% PAGE gel. The bands were blotted onto polyvinylidene difluoride membrane as described in Materials and Methods and sequenced for the first 18 amino acids in the N-terminal region. The two protein bands yielded identical sequences, which would suggest that the subunits contain similar structures in their N-terminal regions. The amino acid sequence of this region was as follows: Met-Asn-Leu-Lys-Ile-His-Pro-Ser-Val-Gly-Ala-X-Leu-Gly-Asn-Arg-Glu-Met-. This N-terminal sequence was compared with previously identified protein sequences present within the Swiss Prot database, and no proteins were found to have similar sequences in either their N-terminal regions or any other region.

The stability of the antibacterial protein. The stability of the D2 antibacterial protein as well as its resistance to changes in heat and pH was assessed. In purified form, the protein could be stored in solution at 4°C for at least 1 month without loss of activity, whereas the unfractionated D2 supernatant lost activity within 9 days when stored under the same conditions, probably because of the presence of proteolytic enzymes. Rou-

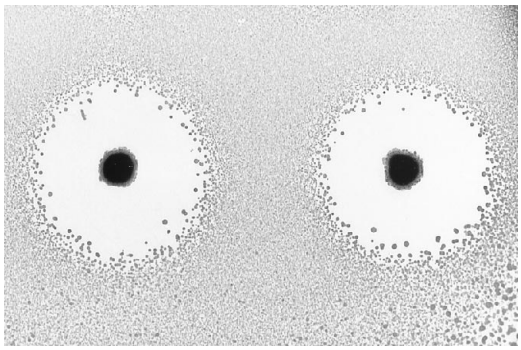

FIG. 3. Autoinhibition around D2 colonies. D2 colonies were grown on VNSS agar plates for 7 days, after which they were overlaid with fresh agar containing D2 cells taken from a 2-day plate culture. Antibacterial activity could be visualized the next day as inhibition zones around the D2 colonies.

tinely, the protein was stored in freeze-dried form at −70°C. There was no detectable loss in protein activity after 1 h of exposure to pH values within the range of 6.5 to 9.5. These data suggest that the protein is resistant to denaturation.

Protein release during the D2 growth curve. In order to determine the stage of growth at which D2 batch cultures produce the antibacterial protein, 50-ml samples of growth medium were sampled from a D2 culture in VNSS at various stages during its growth curve. The antibacterial protein was extracted from these samples by concentrating it onto an ion-exchange column in the same way as described in the purification protocol. Concentrated samples were eluted from the column with 0.5 M NaCl, and the antibacterial protein band was visualized by running it on an SDS–8% PAGE gel and staining with Coomassie blue (data not shown). No antibacterial protein was detected until the cells had entered the stationary phase of growth, which would suggest that this protein is released during stationary phase.

The autoinhibitory property of D2. The significance of cell age with respect to the ability of D2 cells to produce an autoinhibitory protein and the resistance of D2 to its own product was investigated by the overlay technique. D2 colonies of different ages on the surface of VNSS agar plates were shown to produce inhibition zones in overlays of fresh VNSS agar inoculated with D2 cells of different ages, a typical example of which is shown in Fig. 3. D2 plate colonies grown for 2, 3, 4, 5, and 7 days were each assayed with a set of overlays, each of which contained cells from one of the colony ages. Inhibition zones increased in size with increasing killer colony age, possibly because of the higher cell content (and thus protein production) of older colonies. Zone size also increased with decreasing overlay cell age, indicating that the cells from older colonies were more resistant to their own product (Table 2).

Relative sensitivity of bacterial isolates to the bacteriocidal effect of the D2 antibacterial protein. The relative sensitivity of the set of 24 marine bacterial isolates from Australia in addition to bacterial strains taken from the culture collection (Department of Microbiology and Immunology, University of New South Wales) was measured by the drop test assay with different dilutions of the purified antibacterial protein. All isolates, except for one of the unidentified marine strains from Australia, were less sensitive to the bacteriocidal effect of the protein than was D2 as shown in Table 3. The data presented here suggest that the antibacterial activity of the 190-kDa protein isolated from D2 is effective against a variety of surface-living marine bacteria, in addition to several isolates from the soil and gastrointestinal environments. The growth of 77% of the total number of strains exposed to the protein was shown to be inhibited by the drop test assay.

TABLE 2. Inhibition zone size around D2 colonies grown for 2, 3, 4, 5, and 7 days on VNSS agar plates overlaid with fresh agar containing D2 cells taken from plate colonies of the same age categories

Killer cell age (days)	Inhibition zone size (mm) for target cell age (days) of:				
	2	3	4	5	7
2	4–5	3–5	5–7	4–5	4–6
3	7–8	6–7	5–9	4–5	5
4	10–12	6–8	7–8	5	4–5
5	9–10	12	5–6	3–5	3
7	12–14	13–15	9–11	7–8	5–6

MBCs and minimum growth inhibitory concentrations. The minimum concentration of antibacterial protein necessary to kill D2 cells in suspension was calculated as 4 μg/ml. *V. anguillarum* was nonrecoverable at 2 μg/ml. In order to compare the concentration of protein necessary to cause cell death with that necessary for growth inhibition, two strains previously shown to be susceptible to the bacteriocidal effect of the protein (by the drop test) were selected. The strains were C3 and S7 from the set of 24 marine strains isolated in Australia. The growth curves of these strains in solution were measured at different dilutions of purified protein along with the dilution at which the cells were nonrecoverable after 24 h. The target cells were taken from log-phase cultures and inoculated into the test wells at time zero. A comparison between the protein concentration necessary to cause cell death and that necessary to cause growth inhibition is shown in Table 4.

DISCUSSION

At present, there are relatively few reports in the literature of large antibacterial proteins compared with the wide variety of smaller antibiotic molecules known today. Of those proteins so far investigated, many are part of the complex, leukocyte-mediated responses of higher organisms (7). One example of a large, antibacterial glycoprotein is that discovered by Kisugi et al. (6), in the albumen gland of the mollusc *Dolabella auricularia* and designated dolabellanin A. This 250-kDa glycoprotein, consisting of four subunits, was found to inhibit the growth of a variety of bacteria in addition to its having antitumor activity, both of which activities were due to the inhibition of nucleic acid synthesis. Another such example is a calcium-dependent galactose-binding lectin of 15 kDa which was iso-

TABLE 3. Relative dilutions of purified antibacterial protein which produced inhibition zones by the drop test assay against a variety of bacterial isolates

Maximum dilution for bacteriocidal effect	Organism(s)
75×	9 marine isolates[a]
25×	6 marine isolates, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Bacillus pumilus*
5×	3 marine isolates, *Bacillus globi*, *Bacillus globi*, *Bacillus subtilis*
1×	D2, 1 marine isolate
Noninhibited	5 marine isolates, *Serratia liquefaciens*

[a] The set of 24 unidentified marine surface isolates from Australia.

TABLE 4. Relative concentrations of purified antibacterial protein which result in growth inhibition and cell death of the marine isolates C3, S7, and D2 in liquid culture

Isolate	MIC	MBC
D2	1×	4×
C3	1×	>400×
S7	4×	40×

lated from the tunicate *Polyandrocarpa misakiensis* and was shown to have strong antibacterial activity (13).

The 190-kDa antibacterial protein produced by D2, like the *Dolabella* protein, consists of more than one subunit. Kisugi et al. (6) suggested that the similarity of the N-terminal region of dolabellanin to that of several antibacterial peptides may indicate that they are all members of a widely conserved class of antibacterial proteins. The N-terminal region of the D2 protein displayed no close homology to any protein sequences found within the Swiss Prot database. These data, in addition to the fact that no reports of similar large antibacterial proteins of bacterial origin were found in the literature, would suggest that the D2 protein represents a novel antibacterial agent.

In this study, D2 was the only bacterium out of a variety of marine and medical isolates to produce antibacterial products active against the target organisms *V. anguillarum* and D2 when prepared under similar high-density growth conditions. Only D2 was autoinhibitory under these conditions. This would indicate that the D2 antibacterial protein is not a common bacterial product and that this form of autoinhibition is a unique property of the D2 bacterium. The potential impact of D2 upon the bacterial composition of a surface ecosystem was investigated by looking at the concentration of protein required to kill or inhibit the growth of other bacteria. The active concentration of D2 protein depended on the target organism in question, the bacteriocidal concentration ranging, according to drop test assays, by approximately 150-fold between the most sensitive organism (*V. anguillarum*) and the most resistant or unresponsive strain. The D2 antibacterial protein was found to kill *V. anguillarum* cells at a concentration of 2 μg/ml. This is similar to the toxicity of the tunicate lectin discussed above (13), which was shown in preliminary tests to be antibacterial at a concentration of 1 μg/ml. The *Dolabella* protein was reported by Kisugi et al. (6) to be growth inhibitory towards *E. coli* at a concentration of 0.1 μg/ml but was not bacteriocidal whereas the D2 protein was shown to be bacteriocidal at high concentrations and growth inhibitory as its concentration was reduced.

When growth rates in liquid culture in the presence of purified protein were compared between D2 and two other unidentified strains, it was found that, although the bacteriocidal protein concentration was much higher for the unknown strains (10-fold higher for S7 and >100-fold for C3), the concentrations resulting in growth inhibition were equal to or only four times higher than that required for D2 (Table 4). On the basis of these data, it seems likely that any beneficial effects that the presence of this protein may have upon the survival of D2 in competition with other bacterial species would be seen at growth inhibitory concentrations rather than the higher bacteriocidal levels measured by the drop test assay. Despite its production of a self-inhibitory protein, the D2 bacterium is clearly able to survive at high densities in colonies on agar plates. The results of overlay assays with killer and target D2 cells of different ages (Table 2) suggest that the cells become more resistant to their own antibacterial product as they progress further into stationary phase, which may explain the ability of D2 to survive despite the production of an autoinhibitory factor. The potential of D2 as a competitive species in the surface ecosystem is demonstrated by its ability to break up established biofilms of other surface-colonizing isolates on glass slides (unpublished observations).

The ability of the marine bacterium D2 to produce an antibacterial agent in addition to its previously reported inhibitory effects upon marine surface-colonizing organisms (5) indicates that this organism has the potential to greatly influence the composition of a marine surface ecosystem. The presence of D2 on the surface of the tunicate from which it was originally isolated could potentially provide this organism with a means of biofouling control. Such a cooperative relationship would indicate that the presence of the D2 bacterium may play a vital role in the solitary lifestyle and possibly survival of the tunicate. There has been evidence to suggest that the seaweed *Delisea pulchra* is able to regulate the bacterial fouling of its surface through the production of secondary metabolites and thus favor bacterial species with particular phenotypic responses which in turn inhibit phenotypic colonization traits in other bacteria (10). The presence of the D2 bacterium on the surface of the tunicate *Ciona intestinalis* could be another example of a complex interaction between a marine bacterium and a higher organism.

The phenomenon of higher organisms utilizing their associated microflora for the production of beneficial secondary metabolites is common in the marine environment, one well-known example being the production of tetrodotoxin by the pufferfish (17). Although there is no direct evidence available, it has been postulated that several brominated secondary metabolites produced by the marine bryozoan *Amathia wilsoni* are produced by associated microorganisms in a similar cooperative relationship (15). Potentially, there are many cases in which products previously attributed to higher organisms may actually be produced through the maintenance of a specialized bacterial flora.

This paper describes the purification and characterization of a novel antibacterial protein which is released by the marine bacterium D2 into its surrounding medium during the stationary phase of growth. The data show that other bacteria may be included in the range of organisms for which D2 produces inhibitory factors. This activity, in addition to the previously reported inhibitory effect exerted by the D2 bacterium upon the settlement and survival of barnacle and tunicate larvae (5) and bryozoan larvae (4) and its inhibitory effect upon the growth of marine algae (3), diatoms, and fungus (unpublished data), would suggest that D2 is an aggressive competitor against a variety of inhabitants of the marine surface environment. It also raises the possibility that the D2 bacterium may represent an example of cooperative behavior between a higher marine organism and its associated microflora.

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