Purification and Characterization of an Antibacterial Substance Produced by a Marine *Alteromonas* Species

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An extracellular inhibitory substance produced by the marine *Alteromonas* strain P-31 (NCMB 2144) was isolated and purified. The inhibitor was a macromolecule with a molecular weight of 90,000 estimated by Sephadex G-100 chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The inhibitory activity was antagonized by proteinase K and β -amylase and inactivated by heating at 80°C for 30 min. The purified substance exhibited two typical absorption bands in the infrared spectrum at 1,650 and 1,075 cm^{-1} , corresponding to peptide linkages and carbohydrate residues, respectively. These findings allowed us to characterize the antimicrobial compound as a thermolabile glycoprotein. The substance exhibited a broad inhibitory spectrum, being active against clinical and environmental isolates from related and nonrelated taxonomical bacterial groups as well as against the producer strain and other similar marine bacterial strains. The inhibitory glycoprotein did not display cytotoxicity toward mammalian and fish cell lines.

Antibiotic production by marine bacteria has been documented for a long time (3, 6, 8, 13, 18, 20, 25). However, there is a paucity of information dealing with the isolation and purification of the active inhibitory substances. The first antibiotics characterized had low molecular weights, were identified as bromopyrrolic compounds, and were in intracellular locations (1, 7, 15, 17). The reports concerning the purification of high-molecular-weight antibiotics produced by marine bacteria are controversial (4, 5, 13). Although all these antibiotics share a polyanionic character, the nature of these substances has not yet been precisely elucidated.

We have studied the antimicrobial activities of chromogenic marine strains isolated from seawater (30; J. L. Barja, Ph.D. dissertation, University of Santiago, Santiago, Spain, 1979) and from intertidal green and brown algae (10, 22) from the northwest coast of Spain. The strains constituted a homogeneous group belonging to the genus *Alteromonas* (23), and some of them were deposited in the National Collection of Marine Bacteria, Aberdeen, Scotland. Although all bacteria produced extracellular antibacterial compounds, interesting differences were observed. Whereas the strains isolated from seaweeds produced thermolabile, low-molecular-weight inhibitors, a group of *Alteromonas* spp. isolated from seawater produced high-molecular-weight inhibitors. The present work represents the first study in which the precise nature, properties, and inhibitory spectrum of a high-molecular-weight-compound produced by a marine *Alteromonas* sp. was investigated in detail. The possible ecological function and applications of the antimicrobial substance are also discussed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The marine strain P-31 (NCMB 2144) was selected from the homogeneous group of chromogenic *Alteromonas* spp. isolated from seawater in the Ría de Pontevedra (northwestern Spain). This group proved to have both antibacterial and antiviral activities (30). The bacterium was maintained on marine agar (Difco Laboratories, Detroit, Mich.) slants at 15°C and

frozen in marine broth (Difco) with 20% glycerol. The preliminary screening for production of antibacterial substances was conducted by the method of Gauthier (14) with *Staphylococcus epidermidis* ATCC 12228 as test bacterium.

For the production of inhibitory compounds, *Alteromonas* strain P-31 was cultured in Casamino Acids medium (Difco) containing 10% glucose (pH 8.0) at 15°C.

Extraction of the antimicrobial substance. Previous studies (22; Barja, Ph.D. dissertation) have demonstrated that the activity remains strongly bound to the cells in the group of marine strains producing inhibitory substances; therefore, the extraction of the antimicrobial product(s) was carried out by the method of Ballester et al. (4), with some modifications. Marine strain P-31 was cultured in Casamino Acids medium until the end of the logarithmic phase. Whole cells were harvested by centrifugation at $8,000 \times g$ in an RC5-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) for 15 min and suspended in Tris hydrochloride buffer (pH 8.1) containing 10 mM EDTA and 0.6 M NaCl (50 ml of buffer for 10 g of cells). Cells were washed by shaking for 30 min and were centrifuged at high speed ($60,000 \times g$) for 35 min at 4°C in an OTD-2 ultracentrifuge (Sorvall). This cellular wash was repeated until no residual antibacterial activity was detected in the bacterial pellet. The supernatants were pooled and dialyzed against distilled water for 48 h at 4°C. This dialyzed solution was referred to as crude extract. Ammonium sulfate (enzyme grade; Schwarz/Mann, Cambridge, Mass.) was added to the crude extract to a final concentration of ca. 70% saturation. After 12 to 14 h, the precipitate was recovered by centrifugation at $15,000 \times g$ for 35 min and dissolved in distilled water. The dissolved precipitate was extensively dialyzed against distilled water and was termed crude inhibitor.

In order to determine the possible intracellular location of some component with antibacterial activity, the whole cells harvested by centrifugation were subjected to an osmotic shock by suspension in distilled water 1:50 (wt/vol) for 1 h followed by sonic treatment. After being centrifuged at $8,000 \times g$ for 15 min, the supernatant was precipitated with ammonium sulfate and processed as described above for the crude extract.

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To rule out the presence of some antibiotic component of lipidic nature, total lipid extraction from either the crude extract or the cellular pellet was conducted with chloroform-methanol (1:2 [vol/vol]). The fractions were concentrated under vacuum in a rotavapor.

The protein concentrations of the samples recovered in the extraction and purification procedures were estimated by the method of Lowry et al. (24), with bovine serum albumin as standard, and total carbohydrates were determined by the phenol-sulfuric acid procedure of Dubois et al. (11), with glucose as standard.

Antimicrobial activity. The inhibitory activities of the fractions obtained from the different steps in the extraction and purification processes were assayed by the disk diffusion method with *S. epidermidis* as the test bacterium. Plates of tryptic soy agar (TSA; Difco) were covered with a thin layer of soft medium (0.5% agar) previously inoculated with an 18-h culture of *S. epidermidis* in tryptic soy broth. Paper disks impregnated with the antibacterial substance samples were placed on the seeded plates and, after a diffusion period of 15 to 30 min, were incubated at 37°C for 18 h. Clear zones of inhibition around the disks indicated antibacterial activity.

The specific activity of the antimicrobial substance was determined semiquantitatively by the procedure of Ballester et al. (4), with penicillin G (Sigma Chemical Co., St. Louis, Mo.) as the reference antibiotic. One unit of antimicrobial activity was arbitrarily defined as the quantity of active substance giving rise to the same level of inhibition as 1.05 µg of penicillin G did. Specific activity was the number of units per milligram (dry weight).

Dialysis effect. The ability of the antimicrobial substance to pass through dialysis membranes of 2,000 (Sigma) and 15,000 (Serva, Heidelberg, Federal Republic of Germany) molecular-weight cutoffs was examined basically as previously described (22). Briefly, small amounts of marine strain cell paste scraped from marine agar plates and paper disks impregnated with the crude inhibitory substance were deposited on sterile membranes previously placed onto TSA plates seeded with *S. epidermidis*. After a diffusion period of 1 h, the plates were incubated for 18 h at 37°C; diffusibility was evidenced by a clear inhibition zone found under the membranes after their removal.

Purification of the antibiotic substance. (i) **Stage 1: ion-exchange chromatography.** The crude inhibitor was added to a DEAE-Sephadex A-50 column (1.5 by 35 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 50 mM Tris hydrochloride buffer (pH 8.1). Elution was conducted by applying a linear NaCl gradient of between 0.1 and 0.4 M. The flow rate was 30 ml/h, and the effluent was collected in 3-ml fractions to measure the A_{280} . Fractions were assayed for antimicrobial activity by the disk diffusion method as described above. The active fractions were

pooled, dialyzed against distilled water at 4°C for 12 h, and lyophilized.

(ii) **Stage 2: gel filtration.** Gel filtration was performed with a water-cooled (5°C) column (2.2. by 63 cm) of Sephadex G-100 (Pharmacia) equilibrated with 50 mM Tris hydrochloride buffer (pH 8.1) containing 0.1 M NaCl. Blue dextran was used to determine the column void volume and the homogeneity of column packing. The partially purified active substance dissolved in the equilibrating buffer was applied to the column and eluted at a flow rate of ca. 20 ml/h. Fractions (2 ml) were collected and examined for A_{280} and for antimicrobial activity. The apparent molecular weight of the antibacterial compound was estimated by the Andrews plot method (2) with the following proteins as markers: gamma globulin (160,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), myoglobin (17,000), and cytochrome c (12,400).

Electrophoretic properties. Agarose gel electrophoresis was performed basically as previously described (22) by using 1% agarose type I (Sigma) in Veronal (Winthrop Laboratories, New York, N.Y.) buffer (pH 9.24) for 2 h at 200 V in a Shandon-Southern horizontal apparatus with a CVR-300 power supply (Savant Instruments, Inc., Hicksville, N.Y.).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (21) with 12.5% acrylamide in the separating gel and 3% acrylamide in the stacking gel (the ratio of acrylamide/bisacrylamide was 30:0.8). The running buffer was 25 mM Tris hydrochloride-192 mM glycine (pH 8.3) with 0.1% SDS. Samples of the antimicrobial substance and molecular weight standards were loaded in a slab gel (14 by 16 cm), and electrophoresis was conducted at a constant current of 30 mA. Gels were fixed and stained in isopropanol-water-acetic acid (5:4:1 [vol/vol/vol]) containing 0.1% Coomassie brilliant blue R-250. The glycoprotein staining was also conducted by the method of Zacharius et al. (32) with the periodic acid-Schiff reaction.

The antibacterial activities in the agarose and SDS-PAGE gels were assayed by bioautography. Gels were cut into several strips, washed, and placed on TSA plates previously inoculated with *S. epidermidis*. After 18 h of incubation at 37°C, the migration of the active substance(s) was evidenced by zones of growth inhibition.

UV and infrared spectra. Crude and purified antibiotic substances were scanned under UV light in a Techtron 635 spectrophotometer (Varian, Palo Alto, Calif.), and the infrared absorption spectrum was measured with a 237 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) by using dry film preparations placed on KBr disks.

Temperature stability. The effect of heat on antimicrobial activity was evaluated by treating purified samples at 60, 65,

TABLE 1. Purification of the antimicrobial substance produced by marine *Alteromonas* strain P-31

Fraction	Dry wt (mg)	Total protein (mg) ^a	Specific antimicrobial activity ^b (U/mg [dry wt])	Total antibiotic activity (U)	Yield (%)	Sp act (U/mg of protein)	Relative purification
Crude extract ^c	313.6	90.0	5.7	1,775	100	19.7	1
Ammonium sulfate precipitate (crude inhibitor)	48.0	20.0	9.6	463.5	26.1	23.2	1.2
DEAE-Sephadex A-50	8.0	4.8	30.6	244.9	13.8	51.0	2.6
Sephadex G-100	2.9	1.8	79.3	230.0	12.9	127.8	6.5

^a Quantitated by the method of Lowry et al. (24), with bovine serum albumin as standard.

^b Determined semiquantitatively by the method of Ballester et al. (4), with penicillin G as reference antibiotic.

^c Obtained after treatment of whole cells with Tris hydrochloride buffer containing EDTA and NaCl.

FIG. 1. Elution profile from DEAE-Sephadex A-50 assay of the crude inhibitory substance produced by *Alteromonas* strain P-31. —, A_{280} ; ●, antibiotic activity; ---, linear NaCl gradient.

75, 80, 90, and 100°C in a water bath and at 121°C in an autoclave for 2, 10, 15, and 30 min. The remaining activities of heat-treated samples were measured by the paper disk method on TSA plates with *S. epidermidis* as the test bacterium. Untreated preparations were used as controls.

Effect of enzymatic treatments on antimicrobial activity. The enzymatic effect on the antibacterial activity of strain P-31 was evaluated by using solutions (1 mg/ml) of the following enzymes (Sigma): proteinase K, trypsin, carboxypeptidase, α -amylase, β -amylase, glucose oxidase, glucose-6-phosphate dehydrogenase, β -glucuronidase, dextranase, cellulase, collagenase, hyaluronidase, neuraminidase, lipoxidase, phospholipase *c*, acid phosphatase, alkaline phosphatase, RNase, catalase, and peroxidase.

Purified samples of the inhibitor were incubated with the enzyme solutions for 1 h at 37°C. The residual activities of the samples were assayed by the paper disk diffusion method on TSA plates previously seeded with *S. epidermidis*.

Alternatively, the antagonism test of Kaspari and Klemme (19) was conducted. Paper strips impregnated with each enzyme solution were placed across the producer marine bacterium previously streaked along marine agar plates. The plates were incubated at 37°C for 1 h and overlaid with soft TSA inoculated with *S. epidermidis*. A positive enzymatic effect was demonstrated by the lack of an inhibition halo in the cross point between the streaked bacterium and the enzyme-impregnated paper. Appropriate positive controls to test enzymatic activities under these conditions were used for all enzymes.

Screening of antimicrobial activity. The inhibitory spectrum of the purified substance was investigated by using clinical, environmental, and fish-pathogenic bacteria and yeasts from private donors and from the culture collections of the Hospital General de Galicia, Santiago de Compostela, Spain; the American Type Culture Collection, Rockville, Md.; and the National Collection of Marine Bacteria. The number and origins of these strains are listed in Table 2. The paper disk method was used to assay the antimicrobial activity. Twenty microliters of the purified inhibitory compound diluted to contain 0.5 U of antimicrobial activity was applied to 6-mm-diameter disks. The salinity of the medium and temperature of incubation were adapted for optimal growth of each tested strain.

Cytotoxicity assays. The purified antibacterial substance was tested for cytotoxicity basically as previously described

(29) in the following homoiothermic and fish cell lines: HeLa (human cervix epithelioid carcinoma), L-929 (mouse lung fibroblast), CHSE (chinook salmon embryo), and EPC (epithelioma papillosum of carp). Briefly, cell monolayers grown in 24-well plates (Costar, Cambridge, Mass.) were inoculated with 0.1 ml of serial twofold dilutions of the purified compound and incubated at 37°C (HeLa and L-929 lines) or 25°C (fish cell lines). Total or partial destruction of monolayers within a 2-day period was scored as a positive cytotoxic effect.

RESULTS

Preliminary assays conducted to determine the location of the antibacterial substance(s) demonstrated no intracellular active compound. Although the inhibitor was isolated from the culture medium, a large fraction of activity remained associated with the bacterial cells. The antimicrobial activity was retained by a dialysis membrane with a molecular exclusion limit of 15,000, which indicated a high molecular weight.

FIG. 2. Estimation by the Andrews plot method of the molecular weight of the antimicrobial substance by gel filtration in Sephadex G-100.

FIG. 3. SDS-PAGE of the partially purified inhibitor after ion-exchange chromatography. (A) Gel stained by Coomassie brilliant blue; (B) gel developed by bioautography showing a single inhibitory halo. The arrow shows the position of the antibiotic substance, with its molecular mass in kilodaltons.

The quantitative results of the different steps of the purification of the inhibitory substance produced by *Alteromonas* strain P-31 are summarized in Table 1. We obtained a relative purification of about sixfold and a yield of 13%. The chromatographic pattern in the DEAE-Sephadex A-50 assay revealed that the peak of antibacterial activity was eluted at 0.23 M NaCl, but active fractions were found in a range from 0.17 to 0.29 M NaCl (Fig. 1). No correlation between A_{280} and antimicrobial activity was observed. The active fractions were further subjected to gel filtration, and the molecular weight of the purified antibacterial compound was estimated to be $90,000 \pm 5,000$ (Fig. 2).

Following SDS-PAGE, the inhibitor showed a single inhibition zone by bioautography assays. The Coomassie blue staining of SDS-PAGE gels revealed a strong protein band corresponding to the inhibition halo (Fig. 3).

The minimal concentration of the inhibitor required to detect antimicrobial activity was 0.1 U, which corresponds to 0.78 μ g of protein in the purified compound. The active substance maintained full antimicrobial activity in aqueous solution at 4°C for more than 2 months. However, thermosensitivity studies indicated that the inhibitor was a thermolabile substance. The antibacterial activity remained

unaltered after being held at 60°C for 30 min, started to decrease after being held at 75°C for 15 min, and was totally lost after being held at 80°C for 30 min.

With the 20 enzymes tested, antibiotic activity was suppressed only after treatment with proteinase K, β -amylase, catalase, and peroxidase. The antagonist effects of proteinase and β -amylase suggest a glycoprotein nature for the active molecule. The percentages of total protein and carbohydrate of the purified substance were 62 and 34.5%, respectively.

The purified antimicrobial compound displayed a single protein peak at 225 nm in the UV spectrum, and the infrared spectroscopy showed two characteristic absorption bands at 1,650 and 1,075 cm^{-1} , which correspond to peptide bonds and polysaccharide residues, respectively (28). A wide band indicative of the presence of polar groups in the molecule appeared at 3,350 cm^{-1} (28). The crude active substance showed a similar pattern but also an unidentified band at 1,250 cm^{-1} (Fig. 4).

These findings, together with a lack of antimicrobial activity in a lipid extraction, suggest that there was no substantial lipid fraction in the inhibitory substance.

The active substance exhibited a broad inhibitory spectrum with activity against gram-negative and gram-positive bacteria of both related and nonrelated groups (Table 2). Interestingly, the substance inhibited several important fish pathogens in fresh water and seawater. *Bacillus subtilis*, *Pseudomonas fluorescens*, and the enterobacteria *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Serratia marcescens* were the most resistant bacterial species. The antimicrobial compound had no activity against the yeasts examined. It is noteworthy that *Alteromonas* strain P-31 and other similar antibiotic-producing *Alteromonas* strains were inhibited.

The results from cytotoxicity assays in cell cultures indicated that the inhibitory glycoprotein produced by the marine *Alteromonas* strain P-31 did not cause destruction of monolayers of mammalian or fish cell lines under the test conditions.

DISCUSSION

Marine bacteria showing antibacterial (3, 13, 18, 20, 25), antiviral (12, 27, 30), and antiyeast (6, 31) activities have been described for more than 20 years. However, at present, the literature concerning the isolation and characterization of the inhibitory compounds is scarce. The first substances purified had low molecular weights and were isolated from

FIG. 4. Infrared spectrum of crude (A) and purified (B) antibacterial substances in KBr disks, showing typical absorption bands of peptide linkages (1,650 cm^{-1}) and carbohydrate groups (1,075 cm^{-1}). Numbers on the right also indicate percentages of transmittance.

TABLE 2. Activity against clinical and environmental strains of the antimicrobial substance produced by marine *Alteromonas* strain P-31

Strain	Sensitivity ^a
<i>Staphylococcus epidermidis</i> ATCC 12228	+++
<i>Staphylococcus epidermidis</i> HGG 772	+++
<i>Staphylococcus aureus</i> ATCC 25923	++
<i>Micrococcus luteus</i> ATCC 10240	++
<i>Streptococcus faecalis</i> ATCC 10541	++
<i>Bacillus subtilis</i> ATCC 6633	—
<i>Escherichia coli</i> ATCC 25922	(+)
<i>Escherichia coli</i> K-12 185 ^b	—
<i>Salmonella typhi</i> ATCC 19430	+
<i>Salmonella typhimurium</i> ATCC 14028	+
<i>Enterobacter cloacae</i> ATCC 13047	—
<i>Enterobacter cloacae</i> HGG 100839	—
<i>Enterobacter aerogenes</i> HGG 99512	—
<i>Klebsiella pneumoniae</i> HGG 100824	+
<i>Citrobacter</i> strain HGG 99927	+
<i>Serratia marcescens</i> ATCC 8100	(+)
<i>Serratia marcescens</i> HGG 98533	+
<i>Alcaligenes faecalis</i> ATCC 19018	++
<i>Proteus mirabilis</i> HGG 9925	++
<i>Neisseria meningitidis</i> HGG 981	++
<i>Pseudomonas aeruginosa</i> HGG 92603	+
<i>Pseudomonas fluorescens</i> ATCC 13525	—
<i>Vibrio cholerae</i> ATCC 14035	+
<i>Vibrio harveyi</i> KN 96 ^c	+
<i>Vibrio anguillarum</i> ATCC 19264	+++
<i>Vibrio anguillarum</i> R82 ^b	++
<i>Aeromonas hydrophila</i> ATCC 7966	++
<i>Aeromonas hydrophila</i> 80-A1 ^b	++
<i>Aeromonas salmonicida</i> ATCC 33658	+++
<i>Aeromonas salmonicida</i> ATCC 14174	++
<i>Pasteurella piscicida</i> MP-7801 ^d	+++
<i>Pasteurella piscicida</i> MZS-8001 ^d	++
<i>Alteromonas</i> strain P-31 (NCMB 2144) ^b	++
<i>Alteromonas</i> strain CH-112 (NCMB 2156) ^b	++
<i>Alteromonas</i> strain P-52V (NCMB 2145) ^b	++
<i>Rhodotorula</i> sp. ^b	—
<i>Candida albicans</i> ^b	—

^a Width of inhibition zone: +++, 15 to 20 mm; ++, 10 to 15 mm; +, <10 mm; (+), trace activity; —, no inhibition.

^b From our collection.

^c Provided by R. K. Sizemore, Department of Biological Sciences, University of North Carolina, Wilmington.

^d Provided by T. Kitao, Department of Fisheries, Faculty of Agriculture, Miyazaki University, Miyazaki, Japan.

Pseudomonas bromoutilis by Burkholder et al. (7), from a *Chromobacterium* sp. by Andersen et al. (1), and from a *Pseudomonas* sp. by Goatcher and Westhoff (17). Although the inhibitory spectra of these antibiotics were variable, all of them had in common their bromopyrrolic structures and intracellular locations. More recently, we reported (10, 22) the isolation of extracellular inhibitors with molecular sizes of less than 2,000 from *Alteromonas* strains isolated from seaweeds. The partial characterization of these substances allowed us to rule out their bromopyrrolic nature.

On the other hand, high-molecular-weight antibiotics have been isolated from a marine *Vibrio* sp. by Dogget (9) and from *Alteromonas* spp. and *Chromobacterium* spp. by Gauthier (13), Gauthier et al. (16), Gauthier and Flatau (15), and Bernard and Pettazzi (5). Whereas species such as *Alteromonas citrea* and *Alteromonas rubra* produced only a macromolecular polyanionic substance (5, 14), other species, such as *Alteromonas luteoviolacea*, produced both a diffusible macromolecule and two intracellular low-molecular-weight brominated compounds (15). The purification and

characterization of the high-molecular-weight antibiotics have been controversial topics. In fact, some antibiotics reported first as lipopolysaccharides (13) were further considered as polysaccharides (5). Ballester et al. (4), trying for a better purification of the antibiotic produced by an *Alteromonas* sp. isolated by Gauthier (13), confirmed only the polyanionic character; the precise nature and molecular weight of the compound were not elucidated.

In the present study, we have demonstrated that our *Alteromonas* strain produced only one inhibitory substance found both extracellularly and strongly bound to the cell wall. Therefore, we selected the extraction procedure of Ballester et al. (4) because it can give a crude extract which is free from most of the broth proteins and which has a much more concentrated active substance than that obtained from culture medium. This may be why the relative purification attained for the active substance was not very high (Table 1). Purification of the antimicrobial compound allowed us to determine by gel filtration or SDS-PAGE that the substance was a macromolecule with an estimated molecular weight of about 90,000 (Fig. 2 and 3). The results of the characterization assays indicated that the active substance is likely a glycoprotein with a net anionic charge at pH 8.1.

The inhibitory substance shares some of the properties of a bacteriocin: a nondialyzable compound with a protein nature, sensitivity to proteolytic enzymes, and activity against other similar producer strains. However, unlike bacteriocins, the active molecule exhibits a broad inhibition spectrum, being active against other taxonomically remote species (Table 2). Interestingly, the inhibitory pattern of this glycoproteinlike substance was similar to that found for the low-molecular-weight inhibitors produced by the *Alteromonas* spp. isolated from seaweeds in the same area (10, 22).

Some of the characteristics of these compounds are consistent with ecological functions. They could act as effective controls to maintain the species diversity of microbial ecosystems (8). The activity of the producer P-31 strain against similar bacteria and the autoinhibition phenomenon support this hypothesis. Autoinhibition has also been reported previously in different antibiotic-producing marine bacteria (1, 13, 22). The location of the antibacterial substance linked to cells seems to indicate other possible ecological roles in the marine environment. In fact, it has been suggested that exocellular proteins in gram-negative bacteria remain bound to the cell in the periplasmic space after excretion (26). Therefore, the inhibitors could be released into the environment slowly and continuously, preventing the colonization of the adjacent space by competitors. It has also been reported that antibiotic-producing marine bacteria can contribute to the natural inactivation processes of microorganisms in seawater (13, 27, 30).

In addition to their ecological functions, the biologically active substances produced by the marine bacteria may provide new compounds with as yet unexplored industrial applications. In view of the broad inhibitory spectrum displayed by the antibacterial substance produced by marine *Alteromonas* strain P-31 and the absence of cytotoxicity for mammalian and fish cell lines, this strain might be useful in water depuration plants as well as in the field of pathology in aquaculture.

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