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MARINE BIOTECHNOLOGY

Purification and Partial Identification of Novel Antimicrobial Protein from Marine Bacterium *Pseudoalteromonas* Species Strain X153

Arlette Longeon,[1] Jean Peduzzi,[1] Michel Barthélemy,[1] Sophie Corre,[2] Jean-Louis Nicolas,[3] and Michéle Guyot[1]

[1]*Laboratoire de Chimie et Biochimie des Substances Naturelles, UMR 5154 CNRS, Muséum National d'Histoire Naturelle, USM 502, 63, rue Buffon, 75005 Paris, France*
[2]*Micromer Technopole Brest-Iroise, 29200 Brest, France*
[3]*Laboratoire de Physiologie des Invertébrés, DRV, IFREMER, 29280 Plouzané, France*

Abstract: A marine bacterium, X153, was isolated from a pebble collected at St. Anne du Portzic (France). By 16S ribosomal DNA gene sequence analysis, X153 strain was identified as a *Pseudoalteromonas* sp. close to *P. piscicida*. The crude culture of X153 was highly active against human pathogenic strains involved in dermatologic diseases, and marine bacteria including various ichthyopathogenic *Vibrio* strains. The active substance occurred both in bacterial cells and in culture supernatant. An antimicrobial protein was purified to homogeneity by a 4-step procedure using size-exclusion and ion-exchange chromatography. The highly purified P-153 protein is anionic, and sodium dodecylsulfate polyacrylamide gel electrophoresis gives an apparent molecular mass of 87 kDa. The X153 bacterium protected bivalve larvae against mortality, following experimental challenges with ichthyopathogenic *Vibrio*. *Pseudoalteromonas* sp. X153 may be useful in aquaculture as a probiotic bacterium.

Key words: antimicrobial protein, *Pseudoalteromonas*, probiotic bacteria.

INTRODUCTION

In the last few years, marine microorganisms have emerged as a new source for the discovery of novel biologically active compounds (Fenical, 1993, 1997). Marine bacteria are mainly isolated from sediments, but can also be obtained from open oceans or marine surfaces including live marine organisms (Jensen and Fenical, 1994). Antibiotic production by marine bacteria has been documented for decades (Rosenfeld and Zobell, 1947; Baam et al., 1966). Since these earlier reports many low molecular weight antibiotic peptides have been isolated from marine bacteria (Faulkner, 2001). Few publications have focused on antibiotic proteins from marine bacteria, whereas the bacteriocins produced by terrestrial bacteria are recognized to be an excellent source of antibiotic proteins and polypeptides (Tagg et al., 1976; Konisky, 1982; Klaenhammer, 1988), such as nisin and subtilin. These peptides are considered of significant interest to the food industry (Rayman et al., 1981; Delves-Broughton et al., 1996). Nevertheless, 2 antibiotic proteins

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Corresponding author: Michéle Guyot; e-mail: guyot@mnhn.fr

with molecular masses of approximately 100 kDa were purified from *Alteromonas* strains (Barja et al., 1989; McCarthy et al., 1994), and an oligomeric 190-kDa protein was isolated from an unidentified biofilm-forming marine bacterium D2 (James et al., 1996).

Such antimicrobial strains may be of interest in aquaculture as probiotics or by providing antimicrobial compounds specific for ichthyopathogenic strains. In aquaculture the first probiotics tested in fish were those used for terrestrial animals, even though the results were variable (Verschuere et al., 2000; Gomez-Gil et al., 2001; Olafsen, 2001). Survival of these bacteria in aquatic environment was uncertain, however, and attempts have been undertaken to select probiotic strains from marine environments. To date only some genera have been selected, including *Vibrio*, *Aeromonas*, *Alteromonas* and *Lactobacillus* (Gatesoupe, 1999; Verschuere et al., 2000). Ability of these marine bacteria to enhance health was shown for some species such as fish (Olafsen, 2001), shrimp (Austin et al., 1995), bivalves (Gibson et al., 1998; Ruiz-Ponte et al., 1999). The mechanism of protection has not yet been investigated, but it is probably complex including adhesion to the mucus, competition for an ecologic niche, antibacterial activity, and immune stimulation. Clearly, marine bacteria (Austin et al., 1995; Ringo and Vadstein, 1998; Ruiz-Ponte et al., 1999), represent a great potential reservoir for discovery of antimicrobial peptides and proteins.

In a program devoted to the search for antimicrobial substances and probiotics usable in aquaculture and produced by marine bacteria, we investigated bacteria collected from different substrates on the littoral of Brittany. Our attention was focused on a strain, *Pseudoalteromonas* sp., obtained at St. Anne du Portzic (Brittany, France), where the crude culture exhibited high antimicrobial activity.

Materials and Methods

Isolation and Characterization of the Strain

The marine bacterium designated X153 was selected by 10-fold serial dilutions in marine broth (Difco) of isolates (18 strains) obtained by scratching a pebble, covered by Rhodophyceae and Chlorophyceae, and collected at St. Anne du Portzic (Brittany, France).

Analysis of 16S ribosomal DNA sequence was used to identify the X153 isolate. 16S rDNA was amplified by polymerase chain reaction (PCR) using the universal primers and sequenced. PCR was performed with bacterial 16S rDNA primers *8f* (5'-AGAGTTTGATCCATGGC-3') and 1492R (5'-GTTACCTTGTTACGACTT-3') and *Taq* polymerase (Appligene Oncor). The PCR temperature profile was 95°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 52° for 1 minute, 72°C for 90 seconds, and a final extension step at 72°C for 5 minutes. *Taq* polymerase was removed by chloroform (2 vol), then PCR amplicons were precipitated by 0.6 M polyethylene glycol, 2 M NaCl, and rinsed by 70% ethanol. DNA sequencing was performed by Genome Express (Meylan). The nucleotide sequence of the 16S rDNA from X153 strain has been deposited in the EMBL database under accession number AJ581533.

Search of nucleotide sequence homology of 16S rDNA gene was done using the Blast algorithm (Altschul et al., 1990), and the sequences were aligned using CLUSTAL W (Thompson et al., 1994) and MegAlign programs (Dnastar Inc.). Phylogenetic trees were built with the Phylowin program (Galtier et al., 1996) using the neighbor-joining method. The data set was boostrapped for 500 replications to consider the robustness of the internal nodes.

Different characteristics of the X153 strain were determined including gram stain, motility, pigmentation, and utilization of different carbon sources in Baumann medium (Baumann and Baumann, 1981).

Microbial Strains

X153's spectrum of activity was established against microorganisms including gram-negative and gram-positive bacteria, yeast strains, and the filamentous fungus *Aspergillus niger*. Ten human pathogenic strains were used in this study: *Enterobacter gergoviae* (CIP 105140), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538P), *Staphylococcus epidermis* (CIP 6821), *Propionibacterium acnes*, *Propionibacterium granulosum*, *Candida albicans* (ATCC 10231), *Pityrosporum ovale*, and *A. niger*. Twenty-four marine bacteria strains were also tested, among which 7 ichthyopathogenic *Vibrio* strains (stars), *Bacillus globi* sp. *marinus*, *Cytophaga lytica*, *Cytophaga marinoflava*, *Deleya marina*, *Halomonas elongata*, *Oceanospirillum jannaschii*, *Pseudomonas doudoroffi*, *Pseudomonas nautica*, **Vibrio alginolyticus*, **Vibrio anguillarum* (ATCC 19264), **Vibrio carchariae*, *Vibrio costicola*, *Vibrio damsela*, *Vibrio haloplanktis*, *Vibrio harveyi*, *Vibrio mediterranei*, *Vibrio natriegens*, **Vibrio parahaemolyticus*, **Vibrio pectenicida*, *Vibrio*

*pelagius, Vibrio proteolyticus, *Vibrio splendidus, *Vibrio tapetis (P1)*, and *Vibrio vulnificus*. The strains without reference were from our laboratory collection.

Antimicrobial Assays

A culture of X153 strain (10 ml) was grown in marine broth for 48 hours at 25°C and centrifuged at 4500*g* for 15 minutes. The supernatant was filter-sterilized (0.2 μm), and the pellet was resuspended in 10 ml of sterile water and sonicated in ice 3 times for 30 seconds. The single-layer method was used to measure the antimicrobial activity of the crude culture. This method was a modification of the double-layer method (Schilinger and Lucke, 1980). Briefly, 25 ml of 0.7% agar (w/v) containing marine broth, Mueller-Hinton or Sabouraud medium was inoculated with 250 μl of a suspension of test strains (10^8 cells/ml, measured by optical density at 620 nm), and then poured into plates. Wells (5 mm) were cut in the agar and filled with 50 μl of X153 culture, filtered supernatant, or sonicated cell suspension, each in 6 concentrations: crude, 1:2, 1:4, 1:8, 1:16, and 1:32 dilutions. The plates were examined for growth inhibition after 5 days of incubation at 25°C for *A. niger*, 48 hours of incubation at 25°C for *Vibrio* and yeast strains, and 24 hours of incubation at 37°C for the other gram-negative and gram-positive bacteria. Experiments were performed in triplicate for the human strains and in duplicate for the marine strains.

During the purification procedure, antimicrobial activity was monitored by the disk diffusion method assay (Ruiz-Ponte et al., 1999) with *S. aureus* as the susceptible strain. One arbitrary unit of antibacterial activity is defined as the amount of protein giving an inhibitory diameter of 15 mm. Minimum inhibitory concentration (MIC) against *S. aureus* and *V. anguillarum* was determined using the disk diffusion assay (Mandal et al., 2003). Briefly, disks were loaded with 10 μl of solution containing 0.06 to 4 μg of protein; 0.25 μg was the last value giving an inhibitory zone diameter of 8 mm.

Antimicrobial Protein Purification

The *Pseudoalteromonas* sp. strain X153 was cultivated in 1.4-L batches in marine broth for 48 hours at 25°C in static conditions. After centrifugation (4500*g*, 15 minutes, 4°C), the pellet was collected and the supernatant was freeze-dried. Antibacterial activity was found both in the pellet and in the supernatant; however, for practical reasons, purification of the active compound, termed P-153 was achieved from the bacterial cells.

All the purification steps were carried out at 4°C. The bacterial pellet was extracted once with 100 ml of seawater and 3 times with 100 ml of deionized water followed by centrifugations at 15000*g* for 30 minutes. The crude extract was dialyzed against 0.1 M NaCl with a Spectra/Por 1 dialysis tubing (6–8000 molecular weight cutoff) and then lyophilized. Aliquots (35 mg proteins) of the dialysate was applied onto a Sephadex G 200 column (3 × 35 cm) equilibrated with 25 mM ammonium bicarbonate, 0.1 M NaCl, and eluted with the same solution at a flow rate of 12 ml h^{-1}. The active fractions were pooled, dialyzed against 0.1 M NaCl, and then freeze-dried. Protein mixture from size-exclusion chromatography was fractionated by anion-exchange high performance liquid chromatography (HPLC) on a Mono Q HR5/5 column (Pharmacia), equilibrated with 20 mM Tris/HCl buffer, pH 7.8 (solvent A). Samples in the same buffer were loaded on the column, and the proteins were eluted with a 1 M NaCl, 20 mM Tris-HCl buffer, pH 7.8, as solvent B. A 30-minute linear gradient from 20% to 60% solvent B in solvent A was used at a flow rate of 0.8 ml min^{-1}. Finally the peak containing the antimicrobial activity (0.45 M NaCl) was separated by size-exclusion HPLC on a Superdex 200 HR 10/30 (Pharmacia) equilibrated with 25 mM ammonium bicarbonate, 0.15 M NaCl, and eluted with the same solvent at a flow rate of 0.4 ml min^{-1}. Pure P-153 protein was then desalted by using PD-10 column (Pharmacia) eluted with MilliQ water. During all the chromatography steps, absorbance was monitored at 226 nm.

Molecular Mass Determination

Homogeneity and relative molecular mass under denaturing conditions were determined by analytical sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4% to 15% polyacrylamide gel as described by Laemmli (1970). After electrophoresis proteins were visualized by silver staining and the molecular mass of P-153 was estimated using the MW-SDS 200 kit (Sigma). Purified P-153 protein (50 μg) was subjected to size-exclusion HPLC on a Superose 6 HR 10/30 column (Pharmacia). Separation was performed at a flow rate of 0.3 ml min^{-1} under 25 mM ammonium bicarbonate, 0.1 M NaCl. Absorbance was monitored at 226 nm. Gel filtration molecular mass markers (Sigma) used as standards were apoferritin (443 kDa), alcohol dehydrogenase (150 kDa),

bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). Blue dextran (2000 kDa) was used to visualize the void volume.

Amino Acid Analysis

Samples of P-153 (3 nmol) were hydrolyzed at 110°C under vacuum with 6 M HCl constant boiling (Sigma) for 24 hours. The resulting amino acids were separated on a cation exchange PC6A resin (Pierce) and the *o*-phthaldialdehyde derivatives of amino acids were detected with a Waters 420 fluorimeter. Proline was detected at 254 nm by reverse-phase HPLC of its phenylisothiocarbamate derivatives (Cohen and Strydom, 1988), using a Supelcosil LC18-DB column (0.46 × 15 cm, 3 µm; Supelco) as previously reported (Almeida et al., 2000). The amino acid composition, expressed as a mole percent, represents the average of 2 independent determinations and allowed determination of the amount of purified P-153 protein.

Larvae Toxicity

Two tests were carried out to estimate the probiotic activity and larvae toxicity of *Pseudoalteromonas* sp. X153 strain. In a short assay approximately 2-day-old larvae of Manila clam (*Ruditapes philippinarum*) were distributed in a 24-well culture cell plate (approx. 50 larvae in 3 ml of seawater per well) and exposed to 24-hour cultures of X153 at 4 different concentrations (10^5, 10^6, 10^7, and 10^8 cells/ml). Longer-term effects of the probiotic were tested in cultured scallop (*Pecten maximus*) larvae in which mortality occurs without preventive antibiotic treatment with 4 mg L^{-1} chloramphenicol. Two-day-old larvae were reared in 2-L beakers at 5 larvae/ml according to the method described by Robert et al. (1996). X153 cultured in 1 g L^{-1} casamino acids (CA, Merck) was centrifuged (4500*g* for 15 minutes), rinsed, distributed to larval culture at a final concentration of 10^6 bacteria/ml. One liter of seawater containing X153 (106 ml/L) was exchanged in each culture 3 times per week. This concentration appeared necessary in a preliminary experiment to inhibit pathogenic *Vibrio* isolated from diseased larvae. The size (major length) of a sample of larvae was measured automatically by digital imaging. Mortality was determined by counting live and dead larvae in a sample of at least 200 larvae. For all experiments every treatement was performed in triplicate. The larvae sizes in different batches were compared by one-way analysis of variance (ANOVA) followed by the Fisher PLSD test at 5% significance level. Mortality was often too variable between replicates to be submitted to statistical analyses.

Results and Discussion

The X153 strain was a mobile, catalase-positive, strictly anaerobic, gram-negative bacterium, which formed compact yellow colonies. PCR amplification performed with universal bacterial 16S rDNA primers (8*f* and 1492R) produced a fragment of approximately 1400 bp. The sequence of 16S rDNA from X153 strain was aligned against the nearest Blast sequences using the multiple-alignment CLUSTAL W program. Phylogenetic analysis using 16S rDNA sequences and the neighbor-joining method showed the X153 strain is a member of *Pseudoalteromonas* close to *P. piscicida*, *P. peptidysin*, and *Pseudoalteromonas* sp. named Y (Figure 1). This result clearly indicates that X153 strain is very close to a strain isolated in an estuary in Tasmania (Australia), *Pseudoalteromonas* sp. Y, which displayed an algicidal effect against harmful micro-algae (Lovejoy et al., 1998). The same phylogenetic tree was obtained with maximum likelihood and maximum parsimony methods. Although its 16S rDNA sequence was almost identical to that of *P. piscicida* (99.9% identity), DNA/DNA hybridization would be necessary to confirm that both belong to the same species. Table 1 shows that most characteristics of the X153 strain are identical to those previously reported for *P. piscicida* (Vankateswaran and Dohomoto, 2000; Ivanova et al., 2002) with the exception of the utilization of D-galactose and succinate as carbon source.

The antimicrobial activity of the X153 strain was entirely restricted to bacteria. Thus only weak activity was observed against yeast strains (*C. albicans* and *P. ovale*), and no activity was observed against the fungus (*A. niger*). The X153 strain was not tested against harmful microalgae, but when X153 strain was introduced in axenic culture of *Isochrisis galbana* (var Tahiti) used to feed bivalves, a slow-down (but without breakdown) of the culture was observed (data not shown). In contrast, X153 culture was active against several human pathogenic strains as well as marine bacteria, including the 7 ichthyopathogenic *Vibrio*. Among the 10 human pathogenic strains tested, the growth of 2 strains was not inhibited by X153 (*E. gergoviae* and *A. niger*), and 5 strains were only inhibited by the undiluted X153 culture (*E. coli, P. aeruginosa, S. aureus*, and the yeasts *C. albicans* and *P. ovale*). The growth of 3 strains implied in

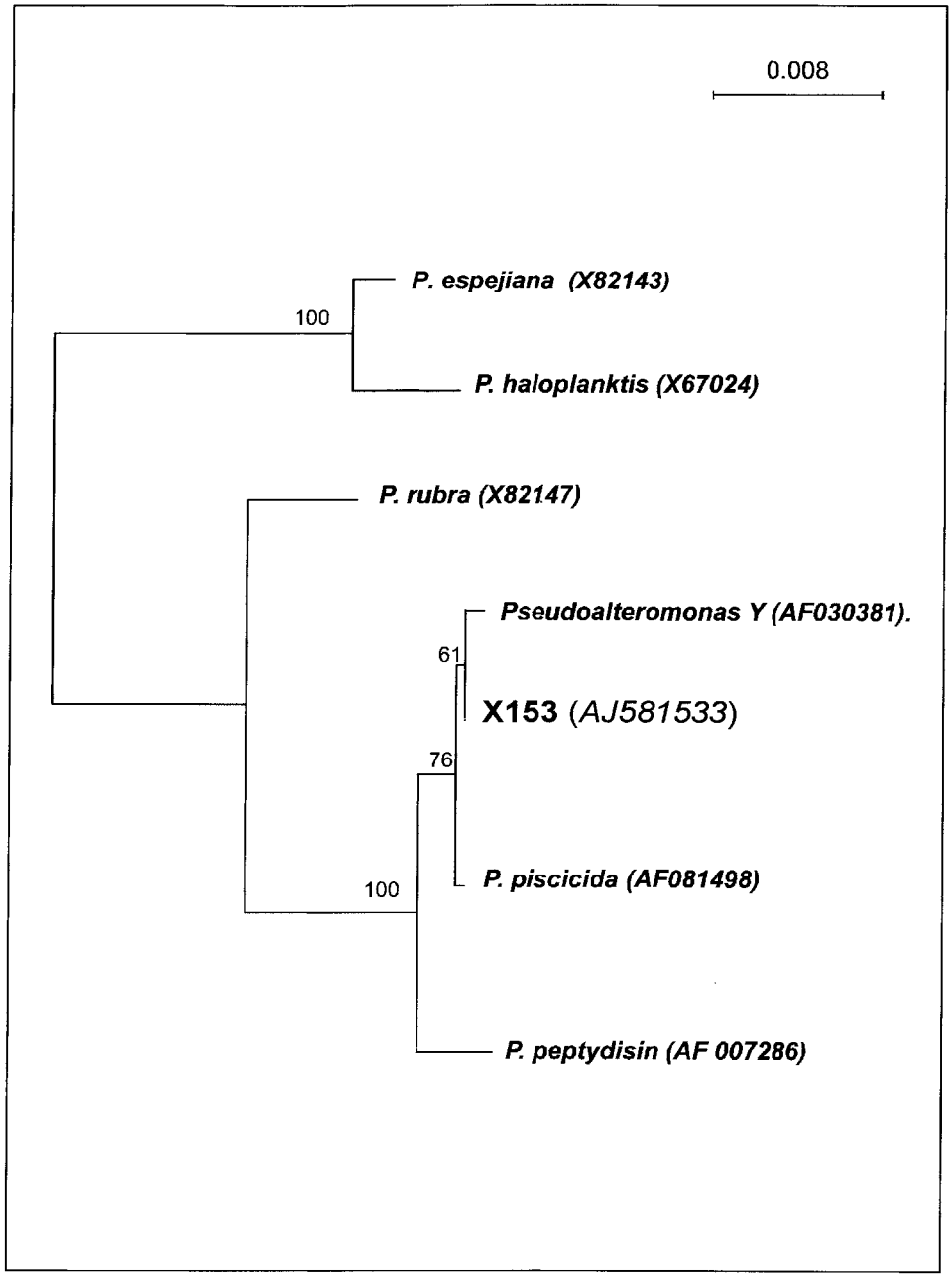

Figure 1. Phylogenetic tree based on 1139-bp sequence alignment of 16S rDNA genes of X153 strain and closely related *Pseudoalteromonas* spp. Tree was constructed by the neighbor-joining method. Boostrap values based on analysis of replicates are indicated at branching points, and bar corresponds to 8 substitutions per 1000 nucleotide positions. The accession number of 16S rDNA sequence from EMBL nucleotide database sequence is indicated in brackets.

dermatologic diseases (*S. epidermidis, P. acnes*, and *P. granulosum*) was inhibited by 1:4 or 1:8 dilutions of X153 clture (Table 2). Antibacterial activity of the X153 culture was markedly stronger against marine strains. Growth of all the marine strains tested was inhibited by X153 culture. Growth of the 7 ichthyopathogenic *Vibrio* strains was inhibited by 1:2 to 1:16 dilutions of X153 culture (Table 3). Tables 2 and 3 also show that biological activity is present in the bacterial cells, but also in the culture supernatant. Comparison of the freeze-dried pellet extract and the freeze-dried supernatant from 1.4 L culture, after dialysis, showed antimicrobial activity in both, but the antimicrobial activity was highest in the pellet. So, we cannot exclude the possibility that another antimicrobial compound is present in the supernatant.

The antimicrobial compound was purified from bacterial cells, until homogeneity, by a 4-step procedure. Crude antimicrobial extracts were obtained from 1 L X153 culture grown in marine broth, and 150 mg of crude protein was routinely extracted from the bacterial pellet (4000 U). After the 3 steps of size-exclusion and anion-exchange chromatography, approximately 300 μg of highly purified P-153 with a specific activity of 600 U/mg was obtained. MIC toward *S. aureus* and *V. anguillarum* was

Table 1. Characteristics of X153 Strain in Comparison with Those of *P. piscicida*

Characteristic	X153	*P. piscicida*[a]
Motility	+	+
Growth at		
4°C	−	−
135°C	+	+
Pigmentation	Yellow	Yellow
Carbon source		
L-Arginine	Weak	−
D-Arabinose	−	−
D-Fructose	−	−
D-Galactose	−	+
D-Mannose	+	+
Melibiose	−	−
Sucrose	+	+/−
Glycerol	−	−
Mannitol	−	−
Sorbitol	−	−
Citrate	+	+
Succmate	+	−

[a]Data from Vankateswaran and Dohomoto (2000) and Ivanova et al (2002).

Table 2. Antimicrobial Activity of X153 Against Some Human Pathogenic Strains[a]

Strain	Whole culture	Supernatant	Sonicated cells
Enterobacter gergoviae	—	—	—
Escherichia coli	1	1	1
Pseudomonas aeruginosa	—	1	—
Staphylococcus aureus	1	1:2	1
Staphylococcus epidermidis	1:8	1:8	1:8
Propionibacterium acnes	1:4	1:4	1:4
Propionibacterium granulosum	1:4	1:4	1:8
Candida albicans	1	—	—
Pityrosporum ovale	1	1	—
Aspergillus niger	—	—	—

[a]A 1 indicates activity without dilution; 1:2, 1:4, 1:8, activity until 1:2, 1:4, 1:8 dilution; —, inactive

approximately 0.3 μM. This antimicrobial activity can be compared to MIC of microcin E492m: 0.04 to 0.08 μM (Thomas et al., 2004).

Protein homogeneity was determined by SDS-PAGE, which indicated a purity superior to 95% and an apparent

Table 3. Antibacterial Activity of X153 Against Marine Bacterial Strains

Strains[b]	Whole culture	Supernatant	Sonicated cells
Bacillus globi sp *marinus*	1:32	—	1:8
Cytophaga lytica	1:32	1:4	1:32
Cytophaga marinoflava	1:8	1:16	1:16
Deleya marina	1:16	1:8	1:8
Halomonas elongata	1:8	1:4	1:4
Oceanospirillum jannaschii	1:2	1:2	1:2
Pseudomonas doudoroffi	1:4	1:8	1:4
Pseudomonas nautica	1:16	1:8	1:8
**Vibrio alginolyticus*	1:2	1:2	1:2
**Vibrio anguillarum*	1:2	1:4	1:4
Vibrio costicola	1:2	1:4	—
**Vibrio carchariae*	1:2	1:2	1
Vibrio damsela	1	1	—
Vibrio haloplanktis	1:32	1:32	1:32
Vibrio harveyi	1:4	1:4	1:4
Vibrio mediterranei	1:8	1:2	1:8
Vibrio natriegens	1:4	1:4	1:4
**Vibrio parahaemolyticus*	1:16	1:2	—
**Vibrio pectenicida*	1:8	1:2	1:4
Vibrio pelagius	1:8	1:2	1:8
Vibrio proteolyticus	1:4	1:2	1
**Vibrio splendidus*	1:4	1:2	1:4
**Vibrio tapetis (P1)*	1:8	1:8	1:8
Vibrio vulnificus	1:4	1:2	1:2

[a]A 1 indicates activity without dilution; 1:2, 1:4, 1:8, 1:16, 1:32, activity until 1:2, 1:4, 1:8, 1:16, 1:32 dilution; —, inactive.
[b]Asterisks indicate ichthyopathogenic strains (aquaculture).

mass of 87 kDa (Figure 2, A). The molecular mass of P-153 was approximately 280 kDa when determined by size-exclusion chromatography on a Superose 6 column (Figure 2, B). This suggests that in aqueous solution, the antimicrobial protein may form trimers. Such a characteristic is similar to the oligomeric structure of the antibacterial protein purified from the marine bacterium D2 (James et al., 1996). The amino acid composition of purified P-153 is shown in Table 4. The P-153 protein was characterized by a high content of aspartic and glutamic acids (near to 26 mole percent) and serine (15.6 mole percent) and exhibited a charged-to-hydrophobic ratio (C/HP; Asx, Glx, His, Arg, Lys to Ala, Pro, Val, Met, Ile, Leu, Phe) of 1.08. The anionic nature of P-153 explains its retention time on the anion-exchange MonoQ column and the 0.45 M NaCl concentration required to elute the protein from the column.

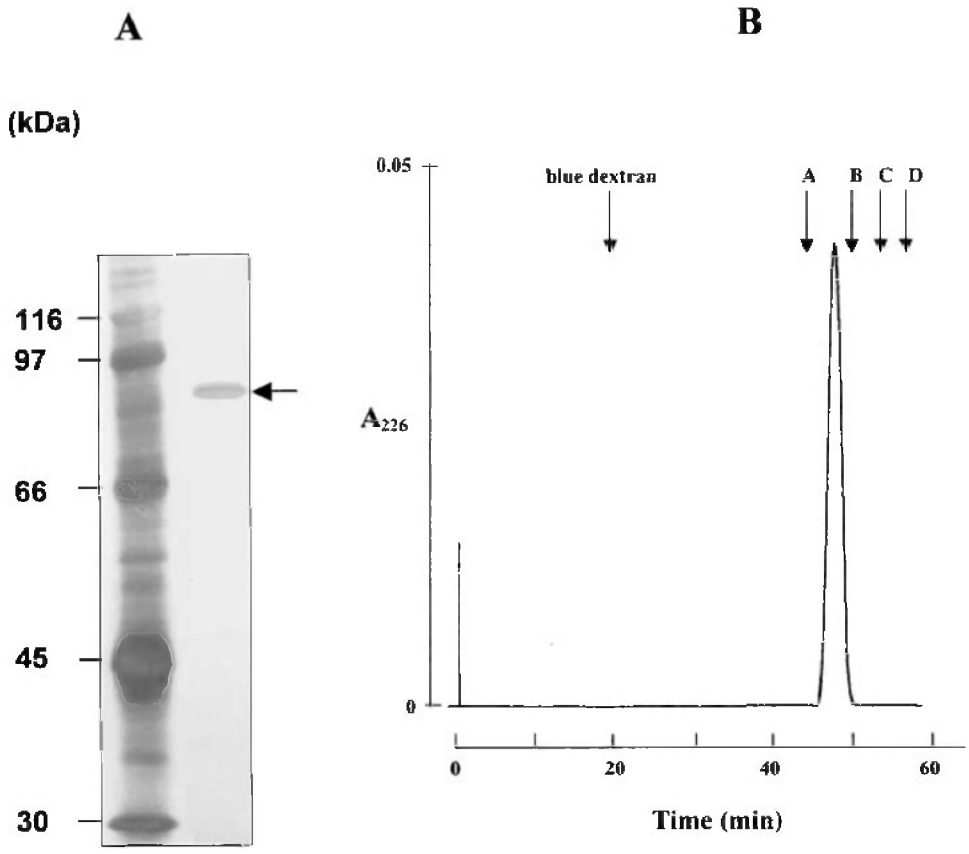

Figure 2. P153 oligomerization. **A**: The purified P153 was loaded onto a 4% to 15% polyacrylamide gel and silver stained. The molecular mass ladder was the MW-SDS200 kit (Sigma). The arrow is pointing to P153. **B**: Size-exclusion HPLC of P153 was performed in 25 mM ammonium bicarbonate, 0.1 M NaCl, on a Superose 6 HR 10/30 column. Separation was performed at a flow rate of 0.3 ml/min, and absorbance was monitored at 226 nm. Molecular mass markers are apoferritin (443 kDa, A), alcohol dehydrogenase (150 kDa, B), serum albumine bovine (66 kDa, C), and carbonic anhydrase (29, kDa, D). Void volume is visualized with blue dextran (2000 kDa).

Table 4. Amino acid composition of P-153

Amino acid	Mole percent[a]	Mol residues/mol[b]
Asx	11.0	94
Thr[c]	5.2	44
Ser[c]	15.6	130
Glx	15.3	129
Gly	14.3	123
Ala	9.4	81
Pro	4.7	41
Val	4.8	42
Met	0.5	4
Ie	3.5	30
Leu	4.3	37
Tyr	2.0	19
Phe	3.0	26
His	2.1	17
Lys	1.7	16
Arg	2.6	22
Trp	ND[d]	—

[a]Amino acid composition was calculated from duplicate 24-hour HCl hydrolysates.

[b]Nearest integer values calculated by assuming a molecular weight of 87,000.

However, when highly purified, the protein P-153 was very unstable. Several attempts to stabilize the protein by addition of salts or bovine serum albumin failed.

The *Pseudoalteromonas* sp. X153 strain was not toxic for bivalve larvae in a short-term assay performed in cell culture plates. Following 24 hours of incubation with X153, the Manila clam larvae exhibited the same behavior as the control until 10^7 cells/ml were added to the larval clam cultures. In a longer-term assay, growth and mortality of scallop larvae treated by X153 strain were measured 16 and 19 days after hatching (Table 5). X153 bacteria protected the larvae against mortality: the mortality rates of larvae were reduced 4-fold in the presence of X153 after 16 days compared with the control without antibiotic. After 19 days, when metamorphosis started, mortality was only 6% in comparison with 28.3% in the control without antibiotic. However, the X153 bacterium reduced the growth rate of larvae approximately 5% at day 16 and 10% at day 19 compared with the controls with or without chloramphenicol (Table 5). The protective effect of X153 appeared efficient, although slightly inferior to that of chloramphenicol. The inhibition of growth observed in these studies, was not necessarily due to the antibacterial protein.

Table 5. Size and Mortality of Scallop (*Pecten maximus*) Larvae Treated by Probiotic Bacteria X153[a]

	Size (μm)		Mortality[b] (%)	
Group	16 days	19 days	16 days	19 days
Control	194.2 ± 3.6	215.9 ± 5.9	20 ± 12.2	28.3 ± 10.1
Control with Cm[c]	196.5 ± 4.6	221.7 ± 4.8	0	0
X153-CA[d]	186.2 ± 3.5	196.3 ± 5.8	5* ± 3.6	6* ± 2.9

[a]Measurements were carried out 16 and 19 days after hatching.
[b]Asterisks indicate mortality significantly different from the control without chloramphenicol.
[c]Control with 4 mg L^{-1} chloramphenicol (Cm) treatment.

Unfortunately, the instability of the purified P-153 protein and the difficulty in obtaining sufficient amounts (>10 mg) prevented direct tests of the P153 protein in cultured scallop larvae. Further experiments will be necessary to determine whether the X153 bacterium will be useful in aquaculture as a probiotic. The X153 bacteria may be more promising for application to shrimp and fish aquaculture than for bivalve larval culture. In conclusion, the high antibacterial activity of the antibacterial protein P-153 against *S. epidermidis* and *Propionibacterium* tested strains suggests an application in human dermatologic diseases. In aquaculture, the broad spectrum of activity of *Pseudoalteromonas* sp. X153 strain, especially against ichthyopathogenic *Vibrio*, allows consideration of its use as a probiotic bacterium in a variety of settings.

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