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

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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.

Keywords: antimicrobial protein - *Pseudoalteromonas* - probiotic bacteria

In the last few years marine microorganisms emerge as a new field for the discovery of novel biologically active compounds from marine origin (Fenical, 1993, 1997). Isolation of bacteria can originate mainly from sediments, but also from open oceans or marine surfaces including marine living organisms (Jensen and Fenical, 1994). Antibiotic production by marine bacteria has been documented for a long time (Rosenfeld et al., 1947; Baam et al., 1966). Since these earlier reports, many low molecular weight antibiotic substances have been isolated from marine bacteria (Faulkner, 2001). In contrast only few publications are dedicated to 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 and are considered of significant interest in the food industry (Rayman et al., 1981; Delves-Broughton et al., 1996). Nevertheless, two antibiotic proteins with a molecular mass 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 have an interest in aquaculture as probiotic or or by providing antimicrobial compounds specific for ichtyopathogenic 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). However survival of these bacteria in aquatic environment was uncertain (or problematic) and attempts have been undertaken to select probiotic strains from marine environment. To date they only belong to some genera including *Vibrio*, *Aeromonas*, *Alteromona*s and lactic acid bacteria (Gatesoupe, 1999, Verschuere et al 2000). Efficiency of these marine bacteria to enhance health were showed for some species such as fish (Olafsen, 2001), shrimp

(Austin et al, 1995), bivalve (Ruiz-Ponte et al, 1999; Gibson et al, 1998). The mechanism of protection has not yet been investigated but it is probably complex including adhesion in mucus, competition for an ecological niche, antibacterial activity, immune stimulation. Marine bacteria (Austin et al., 1995; Ringø and Vadstein, 1998; Ruiz-Ponte et al., 1999), represent certainly a great potential reservoir not sufficiently investigated.

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

MATERIAL AND METHODS

Isolation and Characterization of the Strain.

The marine bacterium X153 was selected by ten-fold serial dilutions in Marine broth (Difco) of an isolate (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 (rDNA) 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 8*f* (5'-AGAGTTTGATCCATGGC-3') and 1492R (5'-GTTACCTTGTTACGACTT-3') and the *Taq* polymerase (Appligene Oncor, Illkirch, France). The used PCR temperature profile was 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 90 s and a final extension step at 72°C for 5 min. *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 company (Meylan, France). 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 were done using the Blast algorithm (Altschul et al., 1990) and then the sequences were aligned using ClustalW (Thompson et al., 1994) and MegAlign programs (Dnastar Inc., Madison, USA). Phylogenetic trees were built with the Phylowin program (Galtier et al, 1996) by using neighbour-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 spectrum of activity was established against microorganisms including Gram-negative and Gram-positive bacteria, as well as 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 seven 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 laboratory collection.

Antimicrobial Assays

A culture of X153 strain (10 ml) in Marine broth for 48 h at 25°C was centrifuged at 4500 *g* for 15 min. The supernatant was filter-sterilized (0.2 µm) and the pellet was suspended in 10 ml sterile water and sonicated in ice three times for 30 s. 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 Lücke, 1980). Briefly, 25 ml of 0.7% agar (w/v) containing Marine broth, Mueller-Hinton or Sabouraud medium were inoculated with 250 µl of a suspension of test strains (10^8 cells per ml, measured by DO at 620 nm), and then poured into plates. Wells (5 mm) are cut in the agar and filled with 50 µl of X153 culture, filtered supernatant or sonicated cell suspension, each in six 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 h of incubation at 25°C for *Vibrio* and yeast strains or after 24h of incubation at 37°C for the other Gram-negative and Gram-positive bacteria. Experiments were made 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 susceptible strain. One unit of arbitrary 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.

Antimicrobial Protein Purification

The *Pseudoalteromonas* sp. strain X 153 was cultivated in 1.4 l batches in Marine broth during 48 hours at 25°C and in static conditions. After centrifugation (4500 *g*, 15 min, 4°C), the pellet was collected and the supernatant freeze-dried. Antibacterial activity was found both in the pellet and 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 seawater and three times with 100 ml deionized water followed by centrifugations at 15000 *g* for 30 min. The crude extract was dialyzed against 0.1 M NaCl with a Spectra/Por 1 tubing (6-8000 molecular weight cut off) and then lyophilized. Aliquot fractions (35 mg proteins) of the dialysate was applied onto a Sephadex G 200 column (3 x 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 min 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^{TM} water. During all the chromatography steps, absorbance was monitored at 226 nm.

Molecular Mass Determination

The homogeneity and the relative molecular mass in denaturing conditions were carried out by analytical polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-15% 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), serum albumin bovine (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 h. 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 x 15 cm, 3 µm; Supelco) as previously reported (Almeida et al. 2000). The serine and threonine contents of the hydrolysates were corrected for partial destruction during the hydrolysis by extrapolation to zero time hydrolysis. The

amino acid composition, expressed as a mole percent, represents the average of two independent determinations and allows to determine 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, about 2-days old larvae of Manila clam *(Ruditapes philippinarum)* were distributed in a 24-well culture cell plate (approximately 50 larvae in 3 ml of seawater per well) and exposed to 24 h-cultured X153 at different concentrations (10^5, 10^6, 10^7 and 10^8 cells per ml). A long-term effect of the probiotic was tested in culture of scallop (*Pecten maximus*) larvae in which mortality occurs without antibiotic preventive treatment by 4 $mg.l^{-1}$ chloramphenicol. Two-days old larvae were reared in 2 l beakers at 5 larvae per ml according to the method described by Robert et al. (1994). The probiotic cultured in 1 $g.l^{-1}$ casamino acids (CA, Merck) was centrifuged (4500 *g* for 15 min), rinsed, distributed to larval culture in a final concentration of 10^6 bacteria per ml and renewed at every change of one liter of seawater (3 times a week). This concentration appeared necessary in a preliminary experiment to produce an antagonism in seawater against pathogenic *Vibrio* isolated from diseased larvae. The size (major length) was automatically measured on a sample of larvae by imager processor. Mortality was determined by counting of alive and dead larvae in a sample of at least 200 larvae. For all experiments every condition was performed in triplicate. Comparisons between the larvae size in different batches were done by one-way analysis of variance (ANOVA) followed by the Fisher PLSD test at 5% significance level. Percentages of mortality were often too different between replicates to be submitted to statistical analyses.

RESULTS AND DISCUSSION

The X153 strain was a mobile, catalase-positive, strict anaerobic Gram-negative bacterium, which forms compact yellow colonies. PCR amplification performed with universal bacterial 16S rDNA primers (8f and 1492R) produced a fragment of approximately 1400 bp. The sequence of 16S rDNA from X153 strain was almost completely determined and aligned against the nearest Blast sequences using the multiple-alignment ClustalW program. Phylogenetic analysis using 16S rDNA sequences and the neighbour-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 the one 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 some 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 crude extract of 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 bivalve, a slow-down (but without breakdown) of T-iso culture was observed (data not shown). The activity of the crude extract of X153 strain was almost restricted to bacteria. Thus, only weak activity was observed against yeast strains (*C. albicans* and *P. ovale*) and no activity against the fungus (*A. niger*). On the contrary, X153 crude culture was highly active against several human pathogenic strains as well as marine bacteria, including the seven ichthyopathogenic *Vibrio*. Among the ten human pathogenic strains tested, two were

not inhibited by X153 (*E. gergoviae* and *A. niger*), five were only inhibited by undiluted suspensions (*E. coli, P. aeruginosa, S. aureus* and the yeasts *C. albicans* and *P. ovale*). Three strains implied in dermatological diseases (*S. epidermidis, P. acnes* and *P. granulosum*) were inhibited until the 1/4 or 1/8 dilution (Table 2). Antibacterial activity of the isolate X153 was markedly stronger against marine strains. All the strains tested were susceptible to X153 crude extract and the seven ichthyopathogenic *Vibrio* strains were susceptible until the 1/2 or 1/16 dilution (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 activity of the freeze-dried pellet extract and the freeze-dried supernatant from 1.4 liter culture, after dialysis, showed antimicrobial activity in both, but the antimicrobial activity was highest in the pellet. So, we can't exclude the possibility of the presence of low-molecular weight compounds in the supernatant.

Hence the antimicrobial compound was purified from bacterial cells, until homogeneity, by a four-step procedure. Crude antimicrobial extracts were obtained from one liter X153 culture grown in Marine broth. From the bacterial pellet, usually 150 mg of proteins were extracted (4000 U). After the three steps of size-exclusion and anion-exchange chromatography, 300 µg of highly purified P-153 were obtained with a specific activity of 600 U/mg. MIC toward *S. aureus* and *V. anguillarum* was 0.3 nM.

Protein homogeneity was determined by SDS-PAGE, which indicated a purity superior to 95% and an apparent mass of 87 kDa (Fig. 2A). When determined by size-exclusion chromatography on Superose 6 column, the molecular mass of P-153 was approximately 280 kDa (Fig. 2B). This suggests that in aqueous solution, the antimicrobial protein likely forms 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 in 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 / 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. However, when highly purified, the protein P-153 was very unstable. Several attempts to stabilize the protein by salts or BSA addition failed.

The *Pseudoalteromonas* sp. X153 strain was not toxic for bivalve larvae in the short-term assay in cell culture plate. After 24 h of incubation with X153, the Manila clam larvae exhibited the same behavior as the control until 10^7 cells.ml^{-1}. In the long-term assay, growth and mortality of scallop larvae treated by probiotic bacteria X153, were measured 16 and 19 days after hatching (Table 5). The probiotic protected the larvae against mortality: the mortality rates of larvae were 4-fold reduced in the presence of X153 after 16 days compared with the control without antibiotic. After 19 days, when metamorphosis started, mortality was only 6% of larvae died in comparison with 28.3% in the control without antibiotic. However the probiotic bacterium reduced the growth rate approximately from 5 % at day 16 to 10 % at day 19 compared with the controls with or without chloramphenicol (Table 5). The probiotic effect appeared efficient, although slightly inferior to that of chloramphenicol. The slow down of growth, was not necessary due to the antibacterial protein. Unfortunately the instability of the purified P-153 protein and the difficulty to purify sufficient amounts (>10 mg) prevented to test it on scallop larvae culture. Additional experiments will be necessary to conclude about the interest of this bacterium as probiotic. It could be more promising for shrimp and fish rearing than for bivalve larval culture. In conclusion, thehigh antibacterial activity of the antibacterial protein P-153 against *S. epidermidis* and *Propionibacterium* tested strains, suggests an

interest in human health for dermatological diseases. On the other hand, in the aquaculture area the broad spectrum of activity of *Pseudoalteromonas* sp. X153 strain especially against ichthyopathogenic *Vibrio*, allows to consider its use as a probiotic bacterium in various rearing.

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Table 1. Characteristics of X153 strain in comparison with those of *P. piscicida*.

	X153	*P. piscicida*[1]
Motility	+	+
Growth at		
4°C	-	-
35°C	+	+
Pigmentation	Yellow	Yellow
Carbone source		
L-arginine	weak	-
D-arabinose	-	-
D-fructose	-	-
D-galactose	-	+
D-mannose	+	+
melibiose	-	-
sucrose	+	- or +
glycerol	-	-
mannitol	-	-
sorbitol	-	-
citrate	+	+
succinate	+	-

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

Table 2. Antimicrobial activity of X153 against some human pathogenic strains

Strains	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	-	-	-

1 : activity without dilution.

1/2, 1/4, 1/8: activity until 1/2, 1/4, 1/8 dilution.

- : inactive.

Table 3. Antibacterial activity of X153 against marine bacterial strains

Strains	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	1/4	1/4
* *Vibrio tapetis (P1)*	1/8	1/8	1/8
Vibrio vulnificus	1/4	1/2	1/2

*ichthyopathogenic strain (aquaculture).

1: 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.

Table 4. Amino acid composition of P-153

	Mole percent[1]	Mol residues / mol[2]
Asx	11.0	94
Thr[3]	5.2	44
Ser[3]	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
Ile	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[4]	-

[1] Amino acid composition was calculated from duplicate 24 h HCl hydrolysates.

[2] Nearest integer values calculated by assuming a molecular weight of 87000.

[3] Values corrected by extrapolation to zero time hydrolysis.

[4] ND, not determined.

Table 5. Size and mortality of scallop (*Pecten maximus)* larvae treated by probiotic bacteria X153. Measurements carried out 16 and 19 days after hatching

	Size (µm)		Mortality (%)	
Experiments	16 days	19 days	16 days	19 days
Control	194.2	215.9	20	28.3
Control with Cm[1]	196.5	221.7	0	0
X153-CA[2]	186.2	196.3	5*	6*

[1] Control with 4 mg l^{-1}chloramphenicol (Cm) treatment.

[2] X153 strain cultured in casamino acids medium.

* Mortality significantly different from the control without chloramphenicol.

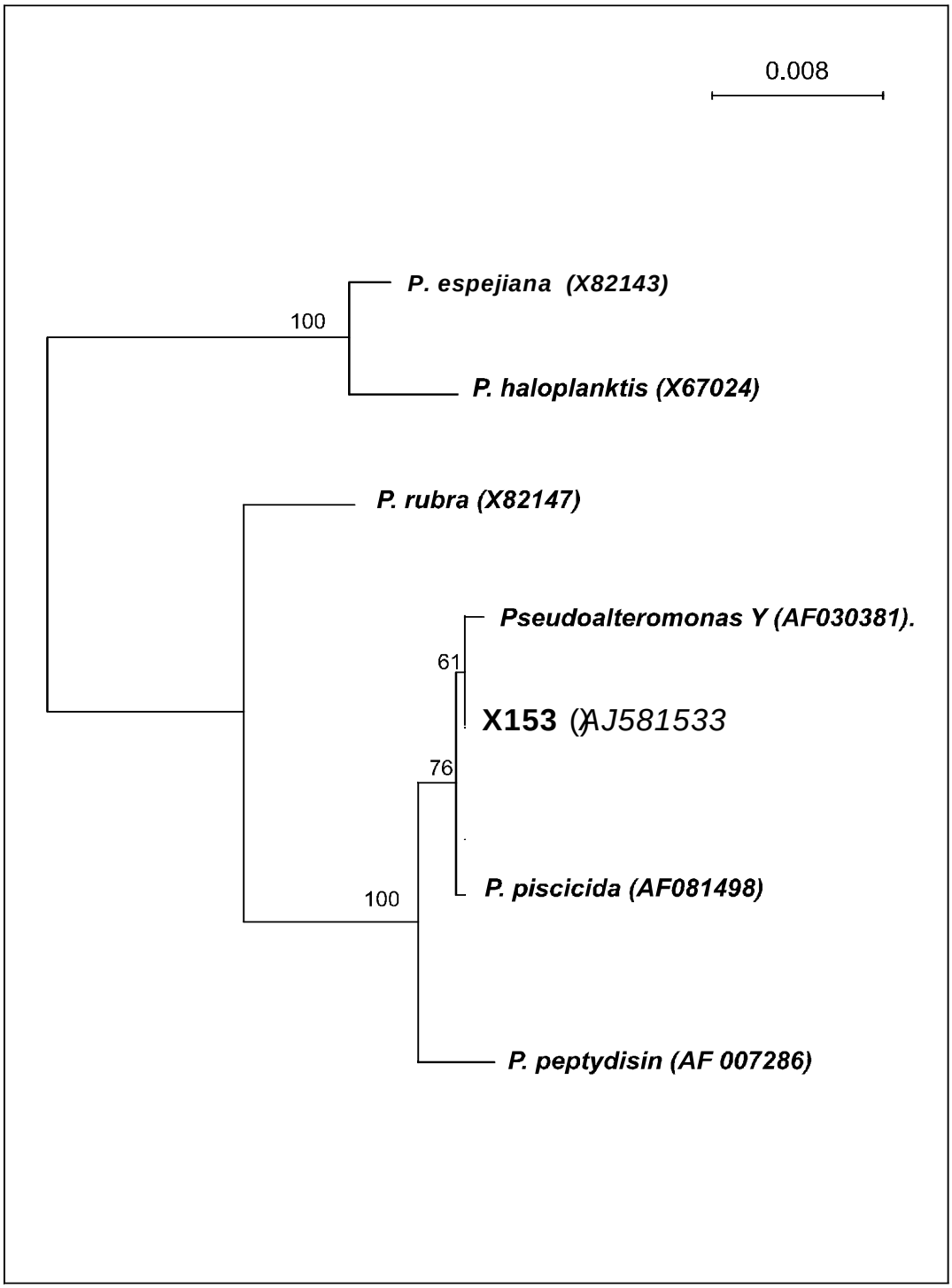

Figure 1 : Phylogenetic tree based on 1139 bp sequence alignment of 16S rDNA genes of X153 strain and closely related *Pseudoalteromonas* spp. Tree was constructed by the neighbour-joining method. Boostrap values based on the analysis of replicates are indicated at branching points and bar corresponds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

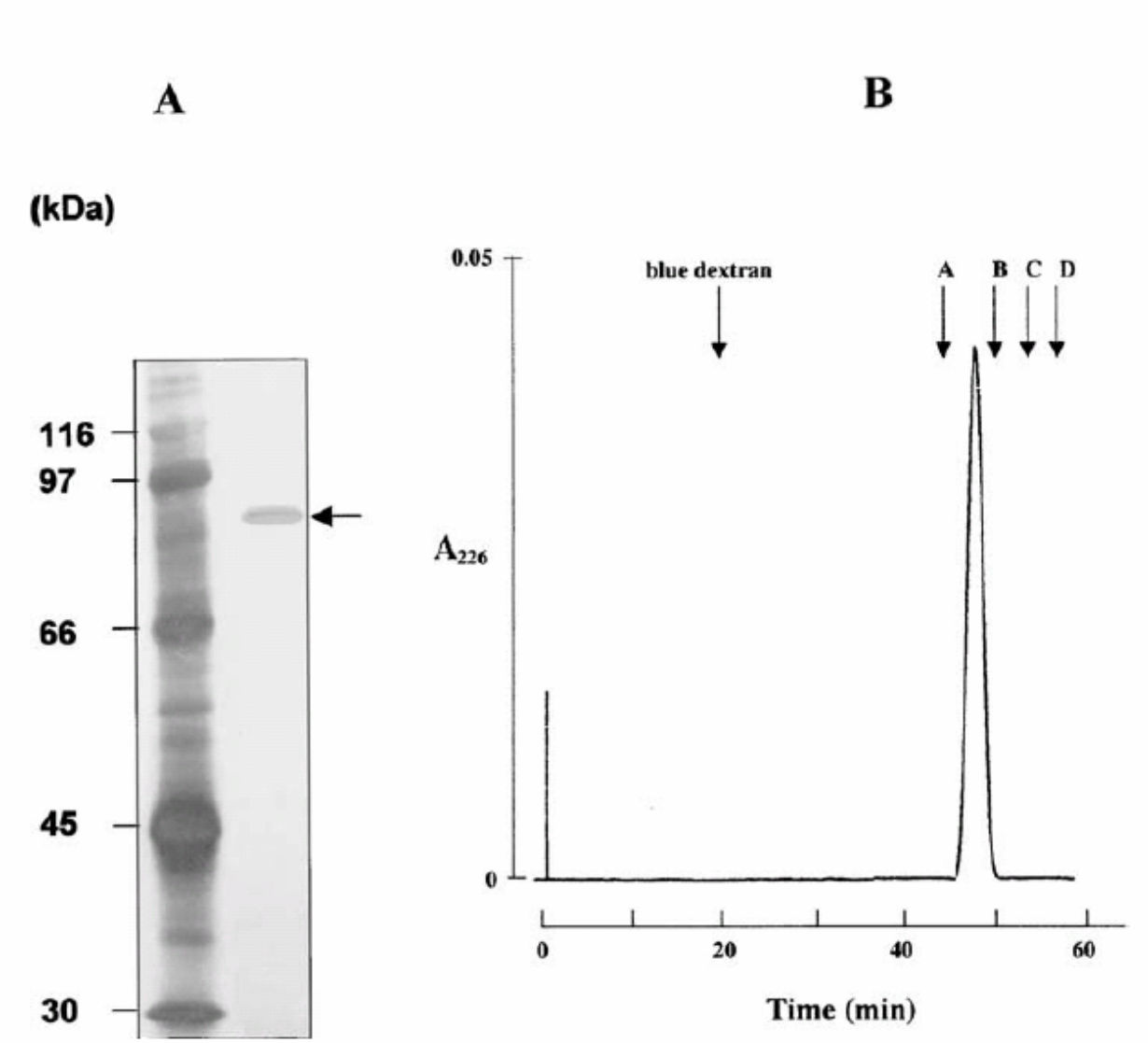

Figure 2. P153 oligomerization.

A: The purified P153 was loaded onto a 4-15% 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)