

# Biofilm formation and proteolytic activities of *Pseudoalteromonas* bacteria that were isolated from fish farm sediments

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## Summary

In order to save natural resources and supply good fishes, it is important to improve fish-farming techniques. The survival rate of fish fry appears to become higher when powders of foraminifer limestone are submerged at the bottom of fish-farming fields, where bacterial biofilms often grow. The observations suggest that forming biofilms can benefit to keep health status of breeding fishes. We employed culture-based methods for the identification and characterization of biofilm-forming bacteria and assessed the application of their properties for fish farming. Fifteen bacterial strains were isolated from the biofilm samples collected from fish farm sediments. The 16S rRNA gene sequences indicated that these bacteria belonged to the genera, *Pseudoalteromonas* (seven strains), *Vibrio* (seven strains) and *Halomonas* (one strain). It was found that *Pseudoalteromonas* strains generally formed robust biofilms in a laboratory condition and produced extracellular proteases in a biofilm-dependent manner. The results suggest that *Pseudoalteromonas* bacteria, living in the biofilm community, contribute in part to remove excess proteinous matters from the sediment sludge of fish farms.

## Introduction

Fish farming is considered an economically feasible and environment-friendly approach to save natural marine

resources. There is considerable interest in exploring smart technologies in this field (van Rijn, 1996). One of the bottlenecks for successful fish farming is lower survival rates of fish juveniles. Because fish fry are weak for diseases and sensitive to environmental changes, management of their viabilities is important to increase the final yield of fish production. In the actual fish farms, there are empirical reports that survival rates of fish fry, such as *Epinephelus bruneus* and *Takifugu rubripes*, are increased when powders of foraminifer limestone are submerged into the fish field or tank (personal communications by Minami-izu Sea-Farming Center, Shizuoka, Japan and Momoshima Sea-Farming Center, Hiroshima, Japan). The exact mode of these treatments is unclear; however, we often observed that the sediments of fish farms were covered with thick biofilms.

Biofilm formation is considered to be a general strategy of bacteria to survive in nature (O'Toole *et al.*, 2000). Unlike planktonic growth in laboratory conditions, natural bacteria sense the change of growth conditions or environmental cues and adhere to the surface of solid phase (conditioning film), where they grow into aggregates and form biofilm. Bacteria cells in the biofilm are usually encased in extracellular polymeric substances and highly tolerable to physicochemical stresses. In addition, drastic changes in the cellular processes are observed in aiming nutrient uptake, cell-to-cell communication, and elimination of potential competitors in the environment. Although biofilm formation is a typical infection style of pathogenic bacteria, several useful traits for human society have been claimed (Morikawa, 2006). For example, the nitrogen removal processes in wastewater treatment are achieved by highly sophisticated microbial cooperation among biofilm cells in activated sludge (Wuertz *et al.*, 2004).

In marine environment, biofilms are also ubiquitous on all aquatic surfaces and having different microbial community structure (Qian *et al.*, 2007). Practical applications of biofilm technology in aquaculture system have been discussed in terms of anti-fouling to deleterious organisms or serving as biofilters to absorb and degrade excess nutrients (Qian *et al.*, 2007). These evidences allowed us to hypothesize that high survival rate of fish fry attributes to beneficial properties of biofilms formed on the artificial surface of submerged limestones. The objectives of this

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study are therefore to isolate useful member bacteria present in the biofilms formed on fish farm sediments and examine their potential properties towards improvement of fish-farming techniques.

## Results and discussion

### Isolation of bacteria

Marine prokaryotes typically grew better in inorganic or oligotrophic media than in complex organic media (Macleod, 1965). It is widely accepted that most natural bacteria are non-culturable in the laboratory conditions and traditional cultivation-based methods are inadequate to study exhaustively microbial diversity in the environmental samples (Thakur *et al.*, 2008). In this study, however, we rather focused on culturable bacteria in aiming to assess biological processes underlying in experimental biofilms and to provide base for developing practical use of them for biotechnological applications.

Natural biofilm samples were collected from the sediments of two sea-farming centres in Japan and further developed in a laboratory condition (Fig. 1A). After several cultivations on a PTM agar plate, bacterial colonies were examined for morphological characters, such as colour, shape, size and surface properties. Fifteen distinguishable strains were isolated and identified by comparative analysis of their 16S rRNA gene sequences (Table 1). A phylogenetical analysis showed that all bacterial isolates belonged to  $\gamma$ -Proteobacteria and closely matched with the genera, *Pseudoalteromonas* (seven strains), *Vibrio* (seven strains) and *Halomonas* (one strain) (Fig. 1B).

Biofilm formation activities of the bacterial isolates were evaluated by the crystal violet (CV) staining method (O'Toole and Kolter, 1998). When each isolate was grown in a 1.5 ml polypropylene tube kept standing, six *Pseudoalteromonas* strains (SB-A1/A2, SB-B1, SB-E1,

SB-G1/G2) and five *Vibrio* strains (SB-G3/G4, SB-H1, SB-I1/I2) formed thick biofilms, but lesser forming abilities were observed for SB-B2, -D1, -E2 and -D2 (Fig. 1C). Although marine biofilm is composed of complex communities of microorganisms and diverse bacterial groups, phylogenetical approaches using exhaustive 16S rRNA gene sequences identified that the most abundant group in association with the marine algae was  $\gamma$ -Proteobacteria, including *Pseudoalteromonas*, *Shewanella* and *Vibrio* (Patel *et al.*, 2003). When plate culture methods were employed, similar  $\gamma$ -Proteobacteria bacteria were dominantly selected from marine biofilms (Lee *et al.*, 2003). Additionally, species of *Pseudoalteromonas* are common bacteria found in association with the surface of biotic and abiotic materials, including shell (Holmström and Kjelleberg, 1999; Dang and Lovell, 2000). This information suggests that isolated bacteria also participate in forming biofilms on the artificially reinforced surface of submerged limestone.

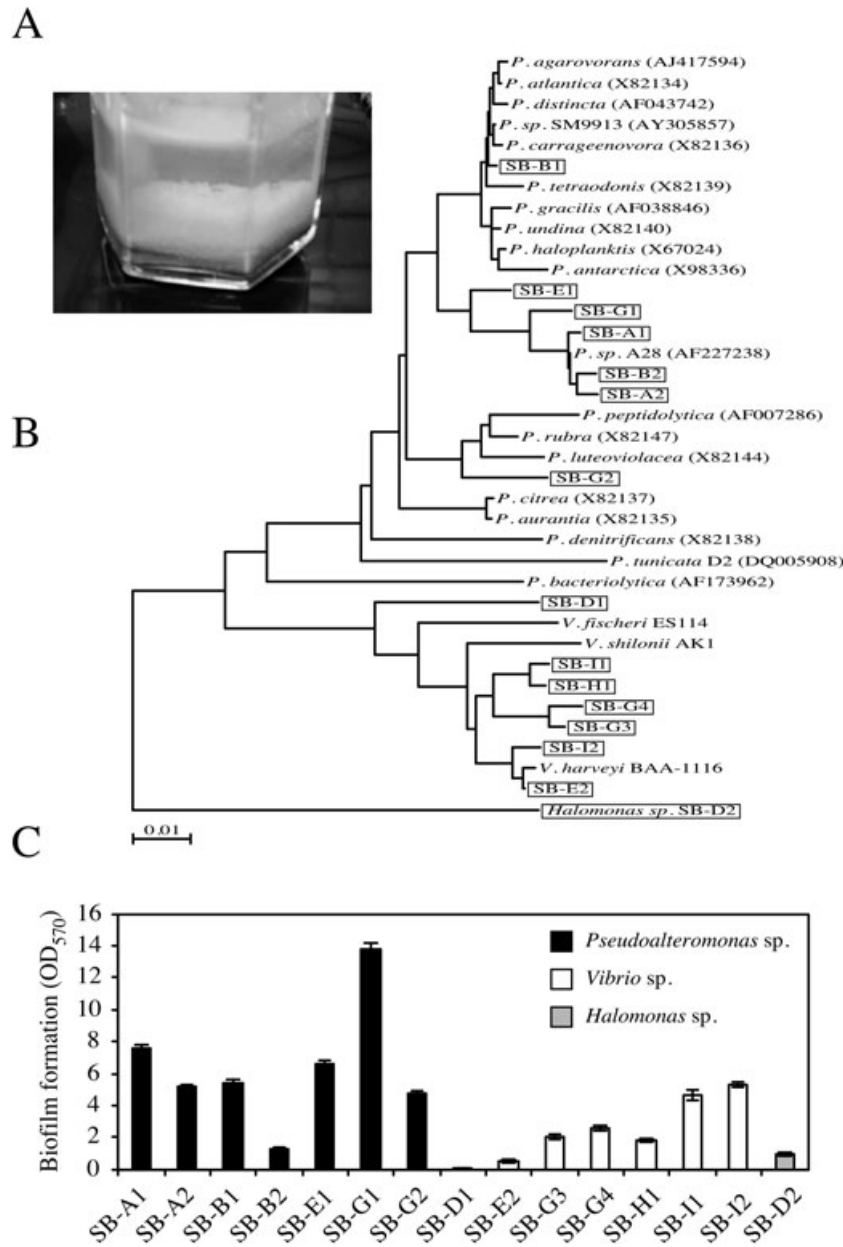
### Protease production by *Pseudoalteromonas* strains

Several species of *Vibrio* are known as primary or opportunistic pathogens of commercially cultured fishes (Tubiash *et al.*, 1970). It was apprehensive that isolated *Vibrio* strains might cause serious fish mortality. On the other hand, many benefits of *Pseudoalteromonas* species have been proposed with regards to anti-fouling of larval settlement, antibiotic effects, and production of many bioactive agents (Holmström and Kjelleberg, 1999). Previous studies showed that *Pseudoalteromonas undina* strain VKM-124 had static activities against pathogenic *Vibrio* sp. and actual application of this strain in aquaculture was concerned (Maeda *et al.*, 1997). Then, *Pseudoalteromonas* strains were selected for the subsequent analyses.

Management of water quality is a critical step in successful fish farming. High concentrations of organic matters derived from remaining foods and faeces of fish

**Table 1.** A summary of bacterial strains isolated from fish farm sediments and their taxonomic identification.

Strain	Collected place	Closely matched species	Max identity (%)	Accession No.
SB-A1	Fish farm sediments,	<i>Pseudoalteromonas ganghwensis</i>	98	AB457045
SB-A2	Momoshima Sea-Farming Center,	<i>Pseudoalteromonas ganghwensis</i>	98	AB457046
SB-B1	Hiroshima, Japan	<i>Pseudoalteromonas elyakovii</i>	99	AB457047
SB-B2		<i>Pseudoalteromonas ganghwensis</i>	97	AB457048
SB-D1		<i>Vibrio parahaemolyticus</i>	94	AB457049
SB-D2		<i>Halomonas venusta</i>	98	AB457050
SB-E1		<i>Pseudoalteromonas aliena</i>	97	AB457051
SB-E2		<i>Vibrio harveyi</i>	98	AB457052
SB-G1	Fish tank sediments,	<i>Pseudoalteromonas ganghwensis</i>	98	AB457053
SB-G2	Minami-izu Sea-Farming Center,	<i>Pseudoalteromonas viridis</i>	96	AB457054
SB-G3	Shizuoka, Japan	<i>Vibrio probioticus</i>	98	AB457055
SB-G4		<i>Vibrio probioticus</i>	99	AB457056
SB-H1		<i>Vibrio parahaemolyticus</i>	97	AB457057
SB-I1		<i>Vibrio harveyi</i>	97	AB457058
SB-I2		<i>Vibrio parahaemolyticus</i>	98	AB457059



**Fig. 1.** Isolation of biofilm-forming bacteria from fish farm sediments.

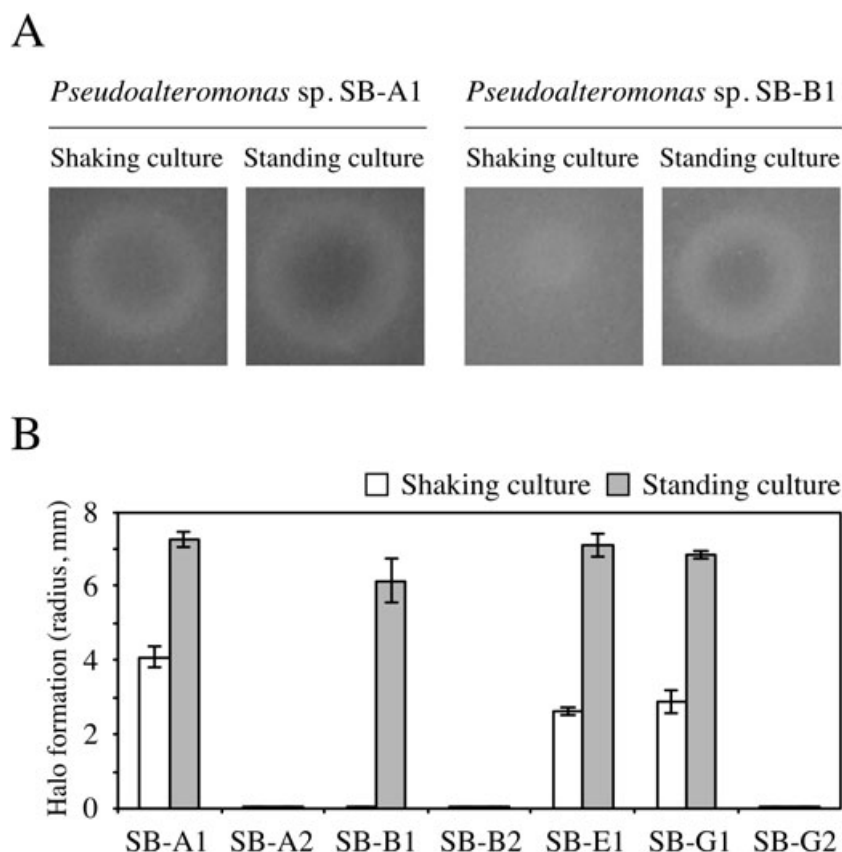
A. Biofilm samples collected from the sediment of fish-farming tank, containing powders of foraminifer limestone (Minami-izu Sea-Farming Center, Japan).

B. Phylogenetical tree of 16S rRNA gene sequences obtained by the neighbour-joining analysis, showing the positions of 15 strains isolated from the developed biofilm samples. Each sequence of relative bacteria is denoted by the strain name and the database accession number. Scale bar, 0.01 substitution per nucleotide site.

C. Biofilm formations by isolated bacteria. Biofilm formations were calculated by the crystal violet staining method. Each tested bacterium is denoted by a strain name under the panel. The error bars indicate the standard errors of the means.

result in a decrease of water quality. This is caused by depletion of oxygen by proliferation of aerobic microorganisms, emission of sulfide and methane by anaerobic bacteria, and outbreak of pathogenic species (Verschuere *et al.*, 2000). In this regard, quick removal of excess nutrients from fish farm sediments is very important. In our phylogenetically analysis, five *Pseudoalteromonas* strains

were clustered with *Pseudoalteromonas* sp. strain A28. SB-B1 was a close relative of *Pseudoalteromonas* sp. SM9913 (Fig. 1B). A28 and SM9913 have been characterized as their productivity of extracellular proteases (Lee *et al.*, 2000; Chen *et al.*, 2002). This suggests that *Pseudoalteromonas* isolates from fish farm sediments might have beneficial traits in primary degradations of



**Fig. 2.** Protease productions by isolated *Pseudoalteromonas* strains.

A. Caseinolytic halo formation by the spot of culture supernatants (5.0 µl) of *Pseudoalteromonas* sp. SB-A1 and SB-B1 on a PTM agar plate supplemented with 1% casein.

B. Specific protease activities in the culture supernatants (5.0 µl) of *Pseudoalteromonas* strains grown at shaking or standing condition (30°C, 2 days). Radius of caseinolytic zones was measured and each value was divided by OD<sub>600</sub> value of the culture. The error bars indicate the standard errors of the means.

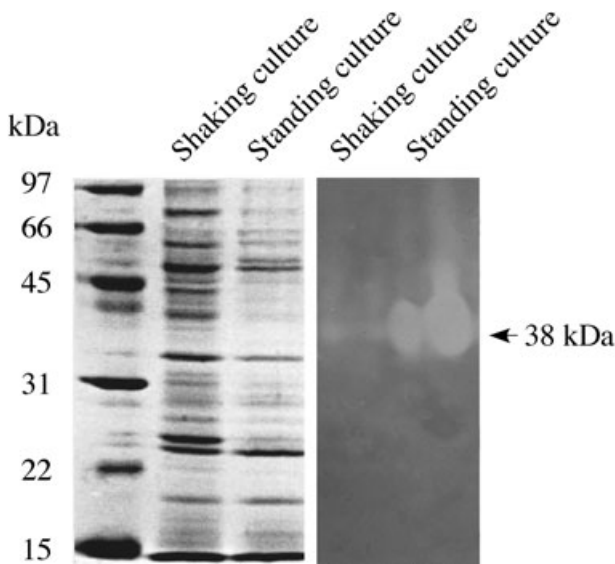
organic matters by producing hydrolytic enzymes including extracellular protease.

To determine whether isolated *Pseudoalteromonas* strains produce extracellular proteases, culture supernatants were spotted on a PTM agar plate containing 1% casein and assayed for protease activities (Fig. 2). Samples were prepared from bacterial cultures grown at shaking (representative of planktonic growth) and standing (preferred by biofilm formation) conditions and their protease activities were compared. Caseinolytic (casein hydrolysing) clear zones were observed around the spots of SB-A1, -B1, -E1 and -G1, demonstrating that these strains were active producers of extracellular proteases. In contrast to moderate inductions of protease activities during standing cultures of SB-A1, -E1 and -G1 (approximately twofold inductions), SB-B1 strictly and significantly produces proteases with response to standing cultivation. Because SB-B1 is phylogenetically distinct from other three strains (Fig. 1C), we hypothesized that SB-B1 possessed unique organization or regulation of protease production accompanied with biofilm formation.

#### *Cloning of the gene encoding a metalloprotease*

For further analysis of extracellular proteases produced by SB-B1, we used zymograph analysis. As shown in Fig. 3, proteins sample prepared from the culture supernatants of SB-B1 showed a major gelatinolytic activity on the zymograph gel at 38 kDa. Intensity of the active band was much lower at shaking condition; whereas, it was apparent when SB-B1 was grown kept standing. These results suggest that biofilm formation of SB-B1 appears to trigger the production of a protease with a molecular mass of 38 kDa. Several extracellular proteases were previously documented in *Pseudoalteromonas* strains (Xiong *et al.*, 2007), where biochemical properties of the SB-B1 protease were well matched with a monomeric type of metalloproteases, such as the protease CP1 of *Pseudoalteromonas* sp. CP76 (38 kDa) (Sánchez-Porro *et al.*, 2003), Empl of *Pseudoalteromonas* sp. A28 (38 kDa) (Lee *et al.*, 2002) and MCP-02 of *Pseudoalteromonas* sp. SM9913 (36 kDa) (Chen *et al.*, 2003). Making the best use of this information, highly





**Fig. 3.** The zymograph analysis in the SDS-PAGE gel. Protein samples prepared from culture supernatants of strain SB-B1 grown at shaking or standing conditions (30°C, 2 days) were separated by a 12.5% SDS-PAGE (left panel). A gelatinolytic band in the zymograph gel indicates protease activities (38 kDa, right panel).

degenerate PCR primers were designed for bacterial metalloprotease genes and used for amplifying the gene encoding an objective protease.

We finally obtained a genome fragment containing entire region of a metalloprotease, designated as Bmpl (biofilm metalloprotease I). The open reading frame of *bmpl* gene was 2184 bp with a GTG start codon and encoded a protein of 727 amino acids (Fig. 4). Amino acid sequence of Bmpl shared similarities with those of the protease MCP-02 of *Pseudoalteromonas* sp. SM9913 (96% identity), Empl of *Pseudoalteromonas* sp. A28 (80%), vibriolysin of *Vibrio parahaemolyticus* AQ3810 (51%), and elastase of *Pseudomonas aeruginosa* PAO1 (50%). The SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), MEROPS (<http://merops.sanger.ac.uk/>) and Pfam (<http://pfam.sanger.ac.uk/>) database searches revealed that the predicted structure of Bmpl was composed of multiple domains, including a signal peptide (M1 to A24), fungalysin/thermolysin propeptide motif (E63 to V112), peptidase propeptide and YPEB (PepSY) domain (A142 to Y199), catalytic domain (G211 to Q354),  $\alpha$ -helical domain (S356 to V500), and two bacterial prepeptidase C-terminal (PPC) domains (E537 to A607, T641 to G713) (Fig. 4A). Bmpl possessed a zinc binding motif (HEXXH) at residues from H345 to H349, followed by a third zinc ligand motif (GXXNEXXSD) at E369, that was, signatures for the M4 subfamily of MEROPS peptidase (thermolysin family).

Although mature Bmpl protease has not been characterized yet, the amino acid sequence of Bmpl beginning

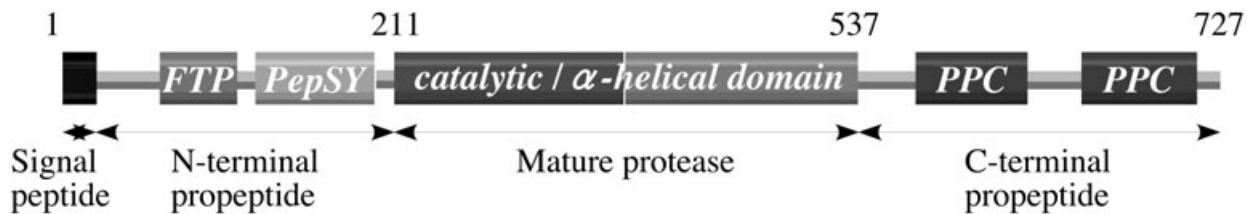
from A205 coincides with the deduced N-terminal sequence of the protease CP1 in strain CP76 (ADATG PGGNQKT; Sánchez-Porro *et al.*, 2003). The predicted protease region of Bmpl has a length of 332 amino acids with a calculated molecular mass of 35 549 Da. This value is somewhat in agreement with that of the detected band in the zymograph gel (38 kDa, Fig. 3).

#### Biofilm formation and protease production

During standing cultivation of SB-B1, biofilm cells were occasionally dispersed in a PTM medium and the cell density was compared with that of shaking cultured cells (Fig. 5). Standing culture exhibited similar exponential growth rate but reached a final cell density lesser than that of shaking culture (Fig. 5A). When an equal volume (5.0  $\mu$ l) of filter-purified culture supernatants was spotted on a casein-containing agar plate, caseinolytic clear zone was observed at later stage of standing cultivation (48 h). However, it was not detectable with shaking cultivation at which culture densities were higher than the standing condition (48 h). It is suggested that growth stages as well as other unknown factors control protease productions in SB-B1. To examine how expression of *bmpl* is regulated in SB-B1 cells, total RNAs were extracted at the different growth stages and subjected to reverse transcriptase PCR analysis (Fig. 5B). The *bmpl* mRNA was abundant at the exponential phase of shaking condition and declined into the stationary phase. During standing cultivation, transcript levels of *bmpl* increased after the onset into the stationary phase and maximized at 2 day. Remarkable accumulation of *bmpl* mRNA seemed to link with higher protease activities in the culture supernatants. This parallelism suggests that the protease activity stimulated by standing cultivation is, at least in part, attributed to the induction of *bmpl* gene expression.

SB-B1 is phylogenetically related with *Pseudoalteromonas* sp. SM9913, which is a cold-adapted strain isolated from deep-sea sediment (Chen *et al.*, 2003). SM9913 produces two extracellular proteases, named MCP-01 and MCP-02. MCP-01 is a novel type of serine protease in the subtilase subfamily (Chen *et al.*, 2007). MCP-02 is a mesophilic metalloprotease that is homologous to Bmpl. At the optimum temperature (12°C), SM9913 mainly produced MCP-01 much more than MCP-02. Purified MCP-01 showed psychrophilic properties and was shown to adapt to the cold environment, whereas role of MCP-02 remained unknown. In this study, we found that the production of Bmpl was considerably induced by the growth condition suitable for biofilm formation. SM9913 and SB-B1 were commonly isolated from sea sediments, where both strains perhaps employed major life style as biofilm. This suggests that

A



B

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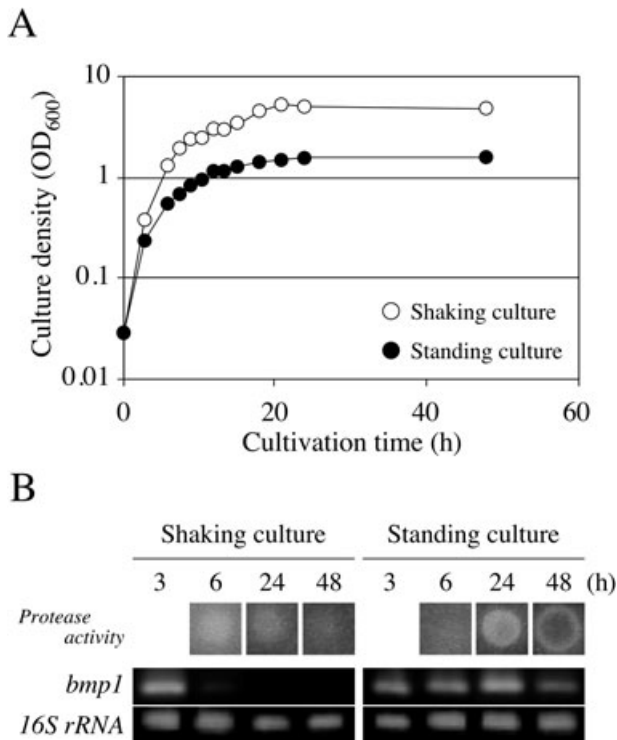
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L T L V Q A T N A V A A N K K Y L N X Q P T I N N M V Q S N S A S L L S V S P N 53
CAGTTGATTGGGTTAAGTGTAGGGAACGAATAGTTGTATTAAGAGGTTTACTAGCAATAACGGAGAAGTAAACACGCCGTTACCAACAAACGTATCAAGGCATTCTGTATTGGTGAT 480
Q L I G L S V G N E L V V L K E F T S N N G E V T R R Y Q Q T Y Q G I P V I G D 93
ACAGTCAGCTTAACATTAACAATGGGATGCTTAAAAAGCCCATGGTCCCGCTGTTTACAACATTGATGAAGATTAGCGGACGTTTCAGCTAAGTTGACAAAAAAGATGCGATTTTA 600
T V S L T F N N G M L K K A H G A A V Y N I D E D L A D V S A K L T K K D A I L 133
AAAGGTCAAAAACAGGCATTGCAGCAAAATCAGTCGGCTGAAAAAACATAACGAGCAATCAAGGCTCGCTATTGGGTTGACGATCAGAATAAGCGCATTAGTTTATGAGGTGCT 720
K G S K T G I A A K S V G L K K H N E Q S R L A I W V D D Q N K A H L V Y E V S 173
TATGTTACTTATGGCAAGTCACCATCAAGACCATATCAGATCATTGATGCTAATACTGGTGGGTTACTTACTAGTACGATACTTACAACATGCCAATGCAACAGGCTCTGGCGGAAAT 840
Y V T Y G K S P S R P Y Q I I D A N T G E V L L S Y D N L Q H A N A T G P G G N 213
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L K T G K Y I Y G T D F D S L D V S Q S G N T C T M N N A N V R T I N L N G G T 253
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Fig. 4. Nucleotide sequence of the gene for the protease Bmpl and its predicted protein structure.

A. Schematic diagram of the domain structure of Bmpl estimated from the SignalIP, MEROPS and Pfam database searches.

B. Nucleotide sequence of the gene for Bmpl and the predicted amino acid sequence. Possible promoter sequences (-35, -10) and a potential ribosome-binding site (SD) are underlined and indicated in lower case. Predicted signal sequence is underlined. A zinc binding motif is shaded and indicated by bold letters. A third zinc ligand motif is shaded and indicated by white-coloured letters. Putative N-terminal residue (A205) and two bacterial pre-peptidase C-terminal (PPC) domains are boxed.



**Fig. 5.** Growth phase and protease productions by *Pseudoalteromonas* sp. SB-B1. A. Growth curves of strain SB-B1 grown at shaking or standing conditions. Cultivated cells were dispersed by vortex and the optical densities (OD<sub>600</sub>) in a PTM medium were determined throughout the growth cycle at 30°C. The experiment was repeated three times and the average value was shown. B. Protease productions in the filter-purified culture supernatants (5.0 µl) and reverse transcriptase PCR analysis of the *bmpI* gene at the different stages of SB-B1 cultivated at shaking or standing conditions. The PCR products in a 1.5% agarose gel were visualized by ethidium bromide staining. The samples of 16S rRNA are indicated to ensure equal loading on the lane.

BmpI-class proteases actively function in the biofilm community.

BmpI is a multiple domain protein consisting of the N-terminal propeptide, the protease region, and the C-terminal propeptide (Fig. 4A). The predicted structure of BmpI provides survival advantages in the biofilm community. The N-terminal propeptide should assist folding of the protein in order to inhibit enzyme activity before secretion and undergoes autoproteolysis during maturation (Yeats *et al.*, 2004). The PPC domain is usually found at the C-terminus of secreted bacterial peptidases and cleaved off after secretion, but retained just prior to activation of the enzyme (Yeats *et al.*, 2003). To date, real function of the PPC domain is unclear; however, several advantages have been proposed for the catalysis of bacterial proteases. Lack of the PPC domain from the metalloprotease in *Vibrio vulnificus* resulted in a loss of proteolytic efficiency to insoluble substrates (Chang *et al.*, 2007).

The PPC domains of the serine protease EspI in *Pseudoalteromonas* sp. A28, which are retained in the mature protease, are supposed to be responsible for its algicidal activity by facilitating the contact with algal cells (Kohno *et al.*, 2007). These previous evidence allows us to concern that PPC domain is involved in an effective contact of the enzyme with a broad range of natural substrates in the environment.

Bacterial metalloproteases have been shown to play important roles in virulence, but they are obviously not unique to pathogenic species in which they display a variety of physiological roles (Häse and Finkelstein, 1993). Natural biofilm is a community structure at high cell population density under poor growth conditions, in which effective uptake of available nutrients is critical for bacterial survival. Although regulatory processes leading to BmpI production have not been elucidated and population of the producing bacteria in natural biofilms is unknown yet, SB-B1 may recruit BmpI along with biofilm formation to utilize surrounding nutrients efficiently, which would contribute in part to remove excess proteinaceous matters from the sediment sludge of fish farms. Further studies are needed to reveal beneficial properties of the isolated bacteria and biofilm communities for future improvement of fish-farming techniques.

## Experimental procedures

### Bacteria strains and growth conditions

Sediment samples, to which powders of foraminifer limestone had already been supplied, were collected from fish farms in Minami-izu Sea-Farming Center (Shizuoka, Japan) and Momoshima Sea-Farming Center (Hiroshima, Japan). Each sample was inoculated into a PTM medium [0.5% Bacto tryptone, 0.5% yeast extract (Difco), 80% artificial seawater (Marine Art, Tomita Pharmaceutical, Tokushima, Japan), pH 7.2], supplemented with powders of foraminifer limestone and incubated for 10 days at room temperature to develop biofilms (Fig. 1A). Biofilm samples were smashed and spread on a 1.5% PTM agar plate. Bacterial strains were isolated and selected on a PTM agar plate, based on the morphological characters of their colonies and tested for biofilm formation. *Escherichia coli* DH5 $\alpha$  was used as a host strain for the general cloning of DNA fragments and was grown in Luria broth medium. Cloning vector pUC18 and pGEM-T EASY (Promega, Madison, WI) were used in *E. coli* DH5 $\alpha$ .

### Phylogenetically analysis of 16S rRNA gene

Whole-cell lysis PCR amplification method was conducted to amplify 16S rDNA, using InstaGene Matrix (Bio-Rad, Hercules, CA). The nearly full-length 16S rRNA gene was amplified by PCR with forward primer (5'-AAGAGTTTGATC ATGGC-3'), reverse primer (5'-AGGAGGTGATCCAACCGC AG-3') and ExTaq DNA polymerase (Takara Bio, Shiga, Japan). PCR products were purified using QIAquick gel



extraction kit (Qiagen, Hilden, Germany) and cloned into pGEM-T EASY. A 16S rRNA gene sequence was determined by BigDye terminator cycle sequencing on an ABI model 3100 DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA), according to the manufacturer's instructions. Sequence data have been submitted into the nucleotide sequence databases under the accession number, SB-A1 (AB457045), SB-A2 (AB457046), SB-B1 (AB457047), SB-B2 (AB457048), SB-D1 (AB457049), SB-D2 (AB457050), SB-E1 (AB457051), SB-E2 (AB457052), SB-G1 (AB457053), SB-G2 (AB457054), SB-G3 (AB457055), SB-G4 (AB457056), SB-H1 (AB457057), SB-I1 (AB457058), SB-I2 (AB457059) respectively. A final data set of 16S rRNA gene sequences was aligned with reference sequences of their close relatives from the public databases. Distance trees based on multiple sequence alignments were performed with ClustalX (version 2.0) (Larkin *et al.*, 2007), using the neighbour-joining method with 1000 bootstrap trials.

### Biofilm formation

Biofilm formation activity on polypropylene was tested as described by O'Toole and Kolter (1998), with some modifications. An overnight culture was diluted  $OD_{600} = 0.3$  and inoculated (1%) into 300  $\mu$ l of PTM liquid medium in a 1.5 ml microcentrifuge tube (TC131615, Nippon Genetics, Tokyo, Japan). The tube was kept standing at 30°C for 2 days. The pellicles and the medium were removed from the tube, which was gently rinsed with distilled water and filled with 500  $\mu$ l of 1% CV solution. After 25 min, the CV solution was removed and the tube was washed with distilled water. The CV attached to the biofilm was dissolved in 400  $\mu$ l of DMSO and quantified by measuring its absorbance at 570 nm. All the data are average of triplicate experiments.

### Protease assays

Bacterial strains were grown in PTM liquid medium at 30°C. After 2 days' cultivation, the cells were removed by centrifugation (8000 g, 5 min) and filtration. The resulting cell free supernatants were used for assay. Aliquot of the sample (5  $\mu$ l) was spotted on a 1.5% PTM agar plate containing 1% milk casein. After an overnight incubation at 30°C, the clear zone surrounded by white zone indicated the extracellular protease activity.

Protease activity was also assessed using zymograph gel electrophoresis (Heussen and Dowdle, 1980). Ammonium sulfate was added to the culture supernatant 80% saturation. The suspension was centrifuged (10 000g, 15 min) and the protein pellet was dissolved into 10 mM Tris-HCl (pH 8.0) and dialysed overnight. SDS-PAGE of the protein sample (50  $\mu$ g each) was performed by the method of Laemmli using 12.5% polyacrylamide gel (Laemmli, 1970). The Gel was renatured in 2.5% Triton X-100 for 1 h and attached with a 12% polyacrylamide gel containing 0.5% gelatin and filter papers. Followed by overnight incubation at 30°C in zymograph developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl<sub>2</sub>, 0.02% Brij35, pH 8.0), gelatin-containing gel was stained with Coomassie brilliant blue R-250 and visualized the clear gelatin-hydrolysed zone with blue colour background.

### Cloning and nucleotide sequencing of the gene for a metalloprotease

To clone the gene encoding a SB-B1 metalloprotease, a 0.6 kb partial gene fragment was amplified using degenerate primers for bacterial metalloprotease genes (forward, 5'-GARATTAAYGGTGCCTAYTC-3'; reverse, 5'-TCACAHGCMGCATCCATBRC-3') and ExTaq DNA polymerase (Takara Bio). Southern hybridization was performed using this 0.6 kb fragment as a probe. The probe was labelled with alkaline phosphatase according to the manufacturer's instructions (AlkPhos direct labelling and detection system, GL Healthcare, UK). The probe hybridized with the 4.8 kb chromosomal DNA digested with HindIII. The DNA fragments corresponding to 4.8 kb were excised from the gel and purified with QIAquick gel extraction kit (Qiagen). These were ligated into the dephosphorylated HindIII site of pUC18, and the ligated plasmids were introduced into *E. coli* DH5 $\alpha$  by electroporation. The library was screened by colony hybridization with the labelled probes as described previously (Sambrook and Russell, 2001). By the screening of ~5000 independent transformants, three positive clones were selected and identified to harbour an identical gene fragment. The nucleotide sequence of the positive fragment was determined and deposited into the public databases under the accession number AB457060.

### Reverse transcriptase PCR analysis

SB-B1 was cultured in a PTM medium at 30°C with either shaking of 120 r.p.m. or standing condition. At the appropriate time, total RNA was extracted from bacterial cells using RNeasy mini kit (Qiagen). cDNA was synthesized from 1  $\mu$ g DNase-treated RNA as described previously (Takei *et al.*, 2008). PCR amplification was performed using each primer set for the metalloprotease (forward, 5'-AACGGAGAAGTAACACGCCG-3'; reverse, 5'-ACCCAAATAGCGAGCCTTGA-3') and 16S rRNA (forward, 5'-GACGGGTGAGTAATGCTTGG-3'; reverse, 5'-TGTGCAATATTCCTCCACTGC-3') genes with KODplus DNA polymerase (Toyobo, Osaka, Japan). PCR condition was 3 min at 94°C, followed by 30 cycles of 15 s at 94°C, 30 s at 58°C and 30 s at 68°C, and 5 min at 68°C. The PCR products were run on a 1.5% agarose gel in 1 $\times$  TBE (89 mM Tris-HCl, 2 mM EDTA) and visualized by ethidium bromide staining.

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