赤潮藻類をピンポイント攻撃する安全かつ実用的なバイオレメディエーターの開発

（課題番号 11555221）

平成 11 年度～平成 14 年度科学研究費補助金（基盤研究（B）(2)）
研究成果報告書

平成 15 年 3 月

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交付金決定額（配分額）

（金額単位：千円）

年　度	直接経費	間接経費	合　計
平成１２年度	2,400	0	2,400
平成 13 年度	1,800	0	1,800
平成 14 年度	1,600	0	1,600
総　計	5,800	0	5,800

研究発表

ポスター発表

Lee S., Kato J., Kuroda, A., Ikeda T., Takiguchi, N., and Ohtake, H.
Cloning and characterization of the *espI* gene encoding algicidal serine protease of the marine bacterium *Pseudoalteromonas* sp. strain A28.
American Society for Microbiology, 2001 5.24, Orlando, Florida, USA.

出版物

Lee, S., Kato, J., Takiguchi, N., Kuroda, A., Ikeda, T., Mitsutani, A. and Ohtake, H.
Involvement of an extracellular protease in the algicidal activity of the marine bacterium *Pseudoalteromonas* sp. strain A28.
Appl. Environ. Microbiol. 66: 4334-4339 (2000).

Lee S., Kato J., Nakashima K., Kuroda A., Ikeda T., Takiguchi, N., and Ohtake H.
Cloning and characterization of extracellular metal protease gene of the algicidal marine bacterium *Pseudoalteromonas* sp. strain A28.
Biosci. Biotechnol. Biochem. 66: 1366-1369 (2002).

加藤純一、李宣沃、大竹久夫、満谷淳
微生物による赤潮藻類の殺藻　Bio Industry, 19(1):36-43 (2002)

Abstract

The marine bacterium *Pseudoalteromonas* sp. strain A28 is able to kill the diatom *Skeletonema costatum* NIES-324. The culture supernatant of strain A28 showed algicidal activity when it was applied to a paper disk placed on a lawn of *S. costatum* NIES-324. The condensed supernatant, which was prepared by subjecting the A28 culture supernatant to ultrafiltration with a 10,000-M_w-cutoff membrane, showed algicidal activity, suggesting that strain A28 produced extracellular substances capable of killing *S. costatum* cells. The condensed supernatant was then found to have protease and DNase activities. Two *Pseudoalteromonas* mutants lacking algicidal activity, designated NH1 and NH2, were selected after *N*-methyl-*N'*-nitrosoguanidine mutagenesis. The culture supernatants of NH1 and NH2 showed less than 15% of the protease activity detected with the parental strain, A28. The protease was purified to homogeneity from A28 culture supernatants by using ion-exchange chromatography followed by preparative gel electrophoresis. Paper disk assays revealed that the purified protease had potent algicidal activity, designated AspI. The purified AspI had a molecular mass for 50 kDa. The optimum pH and temperature of the protease were found to be 8.8 and 30°C, respectively, by using succinyl-Ala-Ala-Pro-Phe- p-nitroanilide as a substrate. The protease activity was strongly inhibited by phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, antipain, chymostatin, and leupeptin. No significant inhibition was detected with EDTA, EGTA, phenanthroline or tetraethylenepentamine.

The determined N-terminal amino acid sequence of purified AspI was identical and internal amino acid sequences showed high similarity with AprI, which is an extracellualr serine protease of marine bacterium *Altermonas* sp. strain O-7. Molecular cloning of the serine protease gene, *aspI*, was performed using the mature AprI encoding DNA fragment as a probe of southern hybridization. The sequencing of

cloned *aspI* gene revealed an open reading frame of 2,073 bp with the capacity to encode a polypeptide of 691 amino acids and with a molecular size of 71,007. The mature AspI is 50 kDa by SDS-PAGE, suggesting that the protease is synthesized as a pre-protein composed of four domains: the signal sequence, the N-terminal pro-region, the mature AspI, and C-terminal extension. The C-terminal regions were characterized by two repeated sequences, which showed high sequence similarities with those of the C-terminal pro-regions from other known bacteria. The predicted product of AspI had 82.9% and 71.5% identity to the mature product (35kDa) and C-terminal pro-region (23kDa) of AprI, respectively. Two repeated amino acid sequences were found in the C-terminal region of AspI. While, the same repeated sequences were in the C-terminal pro-region of AprI. The mature protein of AspI was also predicted to contain only one of the repeated sequences. The *aspI* gene inactivated mutant strain, SP1, was isolated by insertion of kanamycin resistance gene. The algicidal activity of SP1 culture supernatant was dramatically decreased, and this result makes clear that extracellular serine protease AspI of A28 is the major substance for algicidal activity of culture supernatant.

The AspI-inactivated strain SP1 showed still weak algicidal activity. This result suggested that there are some algicidal substances other than AspI in A28 culture supernatant. To investigate another algicidal protease, the metalloprotease gene of A28 was cloned, sequenced and expressed in *E. coli*. The purified metalloprotease, EmpI had molecular weight of 38 kDa and sensitive to metalloprotease inhibitor *1,10*-phenanthroline. Purified EmpI showed algicidal activity when supplied paper disk on the *S. costatum* lawn, but EmpI needs twofold protease unit, which is correspond to 6.2 fold protein concentration of AspI to show algicidal activity. To investigate whether strain A28 produces EmpI protease or not, the protease activities of A28 culture supernatant was analyzed using SDS-skim milk-PAGE gels. The 38 kDa band in extracelluar supernatant of A28 co-migrated with the purified EmpI protease. These results suggest that A28 produced 38kDa metalloprotease corresponding to EmpI.

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Chapter 1.

Introduction

1.1 Background of the present study

1.1.1 Harmful algal bloom phenomena

Occasionally, the algae grow very fast or "bloom" and accumulate into dense, visible patches are appeared near the surface of water. Formerly, "Red Tide" was a common name for such a phenomenon where certain phytoplankton species contain reddish pigments and "bloom" such that the water appears to be colored red. Some kinds of phytoplankton species produce potent toxins, which impact to mass mortalities of wild and framed fish and shellfish, human illness and death from contaminated shellfish or fish, death of marine mammals, seabirds, and other animals, and alteration of marine habitats or trophic structure. Scientists now prefer the term, "Harmful Algal Bloom (HAB)", to refer to bloom phenomena that they cause harm. Unfortunately, the global occurrences of HABs were apparently increased in the over the past decade and coastal countries are heavily impacted by the phenomena of HABs (20).

In marine ecosystem, there are about 5,000 species of phytoplankton were reported in present. In the marine phytoplankton, about 300 species can grow very fast and make blooms and about 40 species of those release some kinds of toxins found in Japanese seawater. When algal blooms occur, most of cases were occupied one kind of phytoplankton species over 95% phytoplankton presence and more than two kinds of phytoplankton were related on the blooms, two kinds of main algal bloom species took possession of over 87% of the phytoplankton in bloomed marine ecosystem (46). The HAB species or potent HAB species were reported such as Cryptomonad,

Cyanobacteria, dinoflagellate, raphidophyte flagellate, gymnodinoids, diatom, haptonema, and aplastidic protozoa (46).

1.1.1 Impacts of HABs

The HAB impacts occur when marine fauna are killed by micro-algal species that release toxins and other compounds into the water, or that kill without toxins by physically damaging or by creating low oxygen concentration conditions as bloom biomass decays. These impacts frequently occur at aquaculture sites where caged fish and shellfish cannot escape the harmful blooms. Farmed fish mortalities from HABs have increased considerably in recent years, and are now a major concern to fish farmer. In Japan, HABs have been often occurred and financial damages on framed fish industries since late in the 1950s. The raphidophyte flagellate, *Chatonella marina* is well-known causative organism of mass mortalities of cultured fish, resulting in 1700 million yen damage in 1972 alone in Japanese seawater (47). In recent years, algal blooms caused by *Heterosigma akashiwo* were occurred in Kagoshima Bay in 1995 and in Nomi Bay in 1997, and 1,090 million and 237 million yen's worth of cultured fishes, respectively, were killed by the blooms (the amounts of damage are estimates made by the Fisheries Agency of Japan). In Hiroshima Bay, *Heterocapsa circularisquama* caused algal bloom, and killed 3,800 million yen's worth of framed oyster in 1998. Today the prediction or prevent of the HABs is one of the most important and urgent subjects in Japan fisheries.

Blooms of macro-algae (seaweeds) can also be harmful, to seagrass and coral reef ecosystems and the food-webs dependent on those habitats. Nuisance seaweed species replace indigenous macro-algae and they thus modify benthic habitats, affect microbial and macro-faunal food webs, and alter key biogeochemical features of costal ecosystem (34).

The public health impact from HABs occurs when toxic phytoplankton are

filtered from the water by shellfish, which then accumulate the algal toxins to levels that are potentially lethal to humans or other consumers (58). These poisoning syndromes are named paralytic, diarrhetic, neuro toxic, and amnesic shellfish poisoning (PSP, DSP, NSP, and ASP) and all of these poisoning syndromes occur within the U. S. and its territories (58).

1.1.2 Interaction of bacteria with HAB species

The increase of global HABs in past decade enhanced efforts to identify factors controlling the population and toxin dynamics of HABs. As the processes of the bloom initiation, maintenance, and decline, as well as toxin production, are dissected into their most basic elements, bacteria and their interactions with HAB species are among the components and processes increasingly cited as potentially important regulators of algal growth and toxicity. It appears that now the question is not whether bacterial-algal interactions play a role in HAB ecology, but instead how these interactions are manifested in terms of population and toxin dynamics and what mechanisms are involved (11). Some bacteria may selectively promote bloom formation by algal species (17), while other bacteria have algicidal effects and are involved in the termination and decomposition of algal blooms (14). It is these cellular level interactions between bacteria and algae, which are among the factors contributing to the regulation of HAB population growth, as well as influencing the successive dominance of the algal community by a given species (15).

Several researchers have isolated the bacteria algicidal to marine algal cells from the costal seawater, sediment, and aquaculture pond and a mass culture of marine diatom (3, 14, 24, 26, 27, 44, 50, 74). The taxa of these marine algicidal bacteria are spread widely among genera; these genera are *Vibrio*, *Flavobacterium*, *Acinetobacter*, *Alteromonas* and *Pseudoalteromonas* as well as gliding bacteria *Saprospira* and *Cytophga*.

In case of bacterial effects on phytoplankton population, algal cells may be affected by extracellular growth inhibiting and algicidal compounds (28), as well as directly attacked by bacteria (24). Direct attacks require cell-to-cell contact between bacteria and algae. For example, *Cytophaga* sp. strain A5Y had algicidal effects on the diatoms *S. costatum, Ditylum brightwell*ii, and *Thalassiosir*a, as well as the raphidophyte *C. antiqu*a, when it was added to algal cultures but not when filtrate alone was added (44). Similarly, *Alteromonas* sp. strains R and S also showed algicidal effects through direct attacks (27). In contrast, *Alteromonas* sp. strains K and D, *Pseudoalteromonas* sp. strain Y, and *Flavobacterium* sp. strain 5N-3 are known to exhibit indirect attacks (14, 27, 38). While algicidal effects were detected with the culture supernatants of these bacteria, physical contact of bacteria with algal cells was not required (14, 27, 38). Indirect attacks are thought to be mediated chemically, and some seem to be species specific. Yoshinaga et al. (76) showed that *Flavobacterium* sp. strain C49 effectively inhibited *H. akashiwo* but did not affect the dinoflagellates or diatoms. However, little is known about the mechanisms of algal lysis by algicidal bacteria at the molecular level.

Yoshinaga *et al.* recently developed a most probable number (MPN) methods for detecting and enumerating algicidal bacteria in seawater and found a close relationship between the dynamics of algicidal bacterial populations and of marine phytoplankton communities (25, 74, 75). An increase or decrease of the number of marine bacteria which inhibit the growth of *Gymnodinium mikimotoi* (dinofallagaete) correlate negatively with the development or decay of a *G. mikimotoi,* respectively, in Tanabe Bay, Japan (74). Moreover, the rapid increase of algicidal bacteria targeting *Heterosigma akashiwo* (raphidophyte flagellate) at the end of *H. akashiwo* blooms which occurred in Hiroshima Bay, Japan, in 1994 and 1995 (31). These results strongly suggest that the marine algicidal bacteria may be one of the agents regulating the dynamics of the phytoplankton community in seawater and also may prove to be useful agents to control the growth of harmful algae in marine

environment.

1.2 Over view of the present study

As the processes of the bloom initiation, maintenance, and decline, as well as toxin production, are dissected into their most basic elements, bacteria and their interactions with HAB species are among the components and processes increasingly cited as potentially important regulators of algal growth and toxicity. To understand population and dynamics of HABs and algicidal bacteria, it is highly desirable to analyze the molecular mechanisms through which algicidal bacteria kill or lyse the algal bloom cells. However, little is known about the mechanisms of the algicidal bacteria at the molecular level.

Pseudoalteromonas species, which are extremely common, slightly halophic, gram-negative bacteria found in many marine ecosystems (62). In this study, the algicidal bacterium *Pseudoalteromonas* sp. strain A28 is investigated. The marine bacterium *Pseudoalteromonas* (formerly named *Alteromonas*) sp. strain A28, which had potent algicidal effects on the diatom *Skeletonema costatum* was previously isolated (3). This organism was also able to kill the diatoms *Thalas-siosira* and *Eucampia zodiacs* and the raphidophyte *Chattonella antiqua.* Algal cells of diatom *Skeletonema costatum* strain NIES-324 are used to examine the strain A28 algicidal activity. Diatoms play an important role as primary producers in marine ecosystems. However, in some Japanese costal waters such as the Ariake Sea and Mikawa Bay, red tides caused by diatoms have frequently occurred in winter and have damaged the production of aqua-cultured seaweed (*Poryphyra* C. Agardh spp.) by competing with it for the utilization of dissolved inorganic nutrients (42). In this study, algicidal mechanism of algicidal marine bacterium is investigated by genetic and biochemical approaches.

The goal of this study is to derive the algicidal mechanism of bacteria, which

would assist to understand the interactions of algal blooms and the bacteria. The key contents of each chapter are as follows.

In chapter 2, algicidal activity of the culture supernatant of *Pseudoalteromonas* sp. strain A28 is described. Genetic information of algicidal activity of strain A28 is investigated comparing with that of algicidal activity deficient mutants of strain A28.

In chapter 3, purification and characterization of the algicidal substance of stain A28 are described. The algicidal substance, which is extracellular serine protease of stain A28, is purified and characterized.

In chapter 4, cloning and analysis of algicidal substance gene from strain A28 chromosomal DNA are carried out. Algicidal protease-inactivated mutant is constructed by insertion mutagenesis on the strain A28 chromosomal DNA, and extracellular protease and algicidal activities are compared with parental strain.

In chapter 5, another algicidal substance of *Pseudoalteromonas* sp. strain A28 other than serine protease is described. The metalloprotease gene is cloned from *Pseudoalteromonas* sp. strain A28 genomic library and expressed in *Escherichia coli*. The algicidal activity of purified metalloprotease is investigated.

Finally, chapter 6 summarizes some important results obtained from this study and suggests future work to clearly understanding the algicidal mechanisms of *Pseudoalteromonas* sp. strain A28.

Chapter 2.

Genetic Investigation of Algicidal Activity of *Pseudoalteromonas* sp. Strain A28

2.1 Introduction

Many workers have discovered the complex interactions within algal blooms and bacteria (11, 30, 49). In the last several decades, it has become well known that some bacteria in lakes, ponds and rivers kill and ingest blue-green algae, and these algicidal bacteria are considered to be one of the agents causing termination of blue-green algal blooms (55, 63, 64, 73). Gliding bacteria of the genera *Myxobacter*, *Lysobacter* and *Cytophaga* were the primary members of these algicidal bacterial populations (72). On the other side, recently, the dynamics of marine bacterial and algal population have been discussions concerning the roles of marine bacteria in HAB and many kinds of algicidal marine bacteria were isolated from where the HAB often occurs (3, 14, 24, 27, 29, 38, 44, 50). It is therefore possible that bacteria having algicidal effects are involved in the termination and decomposition of algal blooms. However, virtually nothing is known about the mechanisms underlying algicidal effects at the molecular level.

A marine bacterium, *Pseudoalteromonas* sp. strain A28, which had potent algicidal effects on the diatom *Skeletonema costatum* was previously isolated (29). The diatom, *Skeletonema costatum* occasionally cause algal bloom in winter at Japanese costal waters such as Ariake Sea and Mikawa Bay. *Skeletonema costatum* bloom damaged the production of aqua-cultured seaweed (*Poryohyra* C. Agardh spp.) by competing with it for the utilization of dissolved inorganic nutrients.

To study the algicidal activity of strain A28 at the molecular level, the algicidal activity deficient mutants were selected and investigated. This chapter describes about genetic and biochemical evidence that an extracellular substances especially, protease is responsible for the algicidal activity of strain A28.

2.2 Materials and methods

2.2.1 Bacterial strain and growth conditions

Pseudoalteromonas sp. strain A28 is an algicidal bacterium isolated from the Ariake Sea of Japan (29). *Pseudoalteromonas* cells were grown at 28°C with shaking in ASWM medium, which was a modified SWM-III medium (6) supplemented with 0.1% Casitone (Difco) and 0.05% yeast extract (Difco). ASWM agar and soft agar were prepared by adding 1.5 and 0.8% agar (Difco) to ASWM medium, respectively.

2.2.2 Algal cultures

The diatom *Skeletonema costatum* (Bacillariophyceae) NIES-324 was obtained from The National Institute for Environmental Studies, Tsukuba, Japan. Clonal axenic cultures were routinely maintained on modified SWM-III medium made with filtered seawater as the base. The seawater was filtered through a 0.45-μm-pore-size nuclepore filter and stored at 4°C in darkness. Cultures were grown at 20°C under an illumination of 35 microeinsteins m^{-2} s^{-1} on a 12-h light-12-h darkness regimen.

2.2.3 Mixed algal-bacterial cultures

A log-phase culture of *S. costatum* NIES-324 was diluted with modified SWM-III medium to a final density of 2×10^5 cells ml^{-1}. Strain A28 cells in the logarithmic phase of growth were added to the *S. costatum* culture to a final density of 10^4 cells per ml, and the mixed culture was further incubated at 20°C with illumination at 35 microeinsteins m^{-2} s^{-1} under a 12-h light-12-h dark regimen for 3 weeks. Enumeration of *S. costatum* cells was accomplished with a differential interference microscope (OPTIPHOT-2 X2F-NTF-21; Nikon Co., Tokyo, Japan). Bacteria were counted with an epifluorescence microscope (OPTIPHOT-2 X2F-EFD2; Nikon) after 4´,6-diamidino-2-phenylindole (DAPI) staining.

2.2.4 Isolation of algicidal activity deficient mutants

Bacterial cells grown overnight in ASWM medium were inoculated into fresh ASWM medium (a 1% inoculum), and the cultures were incubated at 28°C for 4 h with shaking. Cells were then harvested by centrifugation (4,000 × *g*, 10 min, 25°C). Pellets were resuspended in modified SWM-III medium, washed twice with the same medium, and resuspended in 0.3 of the original volume of modified SWM-III medium. Bacterial cells were mutagenized with 50 μg of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) per ml at 28°C for 40 min. The cells were washed twice with modified SWM-III medium, resuspended in ASWM medium, and then incubated overnight with shaking at 28°C. Axenic cultures of *S. costatum* were grown in modified SWM-III medium for 1 week, and 1 ml of the *S. costatum* culture was mixed with 2.5 ml of molten ASWM soft agar (equilibrated to 47°C). The mixture was immediately poured onto an ASWM agar plate. After the agar solidified, mutagenized with NTG bacteria were transferred onto the agar plates with toothpicks, and the plates were incubated at 20°C under an illumination of 35 microeinsteins m^{-2} s^{-1} on a 12-h light-12-h darkness regimen. Bacterial colonies, which failed to produce clear zones on lawns of *S. costatum,* were picked, purified,

and maintained on ASWM agar plates.

2.2.5 Algicidal activity assess

A log-phase culture of *S. costaum* culture was used to investigate algicidal activity of strain A28 culture supernatant. Strain A28 culture supernatant, which were sterilized by being filtered through a 0.2-μm-pore-size polyethersulfone membrane filter (Kurabo, Osaka, Japan) added to *S. costatum* culture then incubated at 20°C with illumination at 35 microeinsteins m^{-2} s^{-1} on a 12-h light-12-h darkness regimen. Algicidal activity judgments were accomplished with a differential interference microscope enumeration of *S. costatum* cells. To paper disk assess of algicidal activity of the strain A28 culture supernatant, *S. costatum* cells were first grown for 4 days on modified SWM-III agar plates to form algal lawns. Strain A28 culture supernatant, which were sterilized by filtering, were applied to 8-mm-diameter paper disks (Advantec Inc.) on the *S. costatum* lawns. The plates were incubated overnight at 20°C under illumination. Algicidal activity was assessed by the presence of clear zones around the paper disks.

2.2.6 Enzyme activity assays of strain A28 culture supernatant

The protease activity of strain A28 culture supernatant was measured as azocasein (Sigma) hydrolytic activity. A28 cultures were grown for 18 h at 28°C in ASWM medium then culture supernatant used for assays. A reaction mixture (0.5 ml) containing 10 mg of azocasein and sample solution appropriately diluted in 50 mM Tris-HCl buffer (pH 7.8) was incubated at 30°C for 30 min. The reaction was stopped by adding 0.5 ml of cold 10% trichloroacetic acid to the reaction mixture. The precipitate was removed by centrifugation at 10,000 × *g* for 5 min, and the absorbance of the supernatant at 400 nm was measured. One unit of protease activity was defined as the amount of enzyme that caused an incremental change of

one absorbance unit per hour. Cellulase and amylase activities were determined by measuring the release of reducing sugars by the dinitrosalicylic acid method (41). The reaction mixture contained 0.1 ml of bacterial culture supernatant and 0.9 ml of 50 mM Tris-HCl buffer (pH 7.8) supplemented with either 2.5% carboxymethyl cellulose or 2.5% soluble starch. The reaction mixture was incubated at 30°C for 1 h, and the reaction was stopped by boiling the mixture for 10 min. DNase activity was determined by the methods of Blaschek and Klacik (4). To determine the DNase activity, 75 ml of a 1 mg ml^{-1} DNA (type XIV; Sigma) stock solution and 725 ml of DNase buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol) were added to a 1.5 ml microcentrifugation tube. After pre-warming of the solution at 30°C for 5 min, 0.1 ml of culture supernatant was added and mixed. The polymerized DNA present at time zero and remaining after 60 min at 30°C was precipitated with 0.1 ml of 5 N HCl. Turbidity was developed at 37°C for 10 min and was estimated by reading the absorbance at 600 nm. DNase activity (1 U) was defined as the amount of enzyme depolymerizing 1 mg of DNA per min at 30°C.

2.3 Results

2.3.1 Mixed cultures of strain A28 and *S. costatum*

S. costatum NIES-324 demonstrated typical batch culture kinetics in modified SWM-III medium (Fig. 2.1). Rapid lysis of the *S. costatum* NIES-324 occurred in the presence of strain A28 cells. When *S. costatum* NIES-324 and strain A28 were grown together, the algal population rapidly declined around 10 days after the start of cultivation. In contrast, bacteria were able to grow in the mixed culture, and the bacterial population increased to up to 1×10^7 cells per ml within 14 days after the start of cultivation.

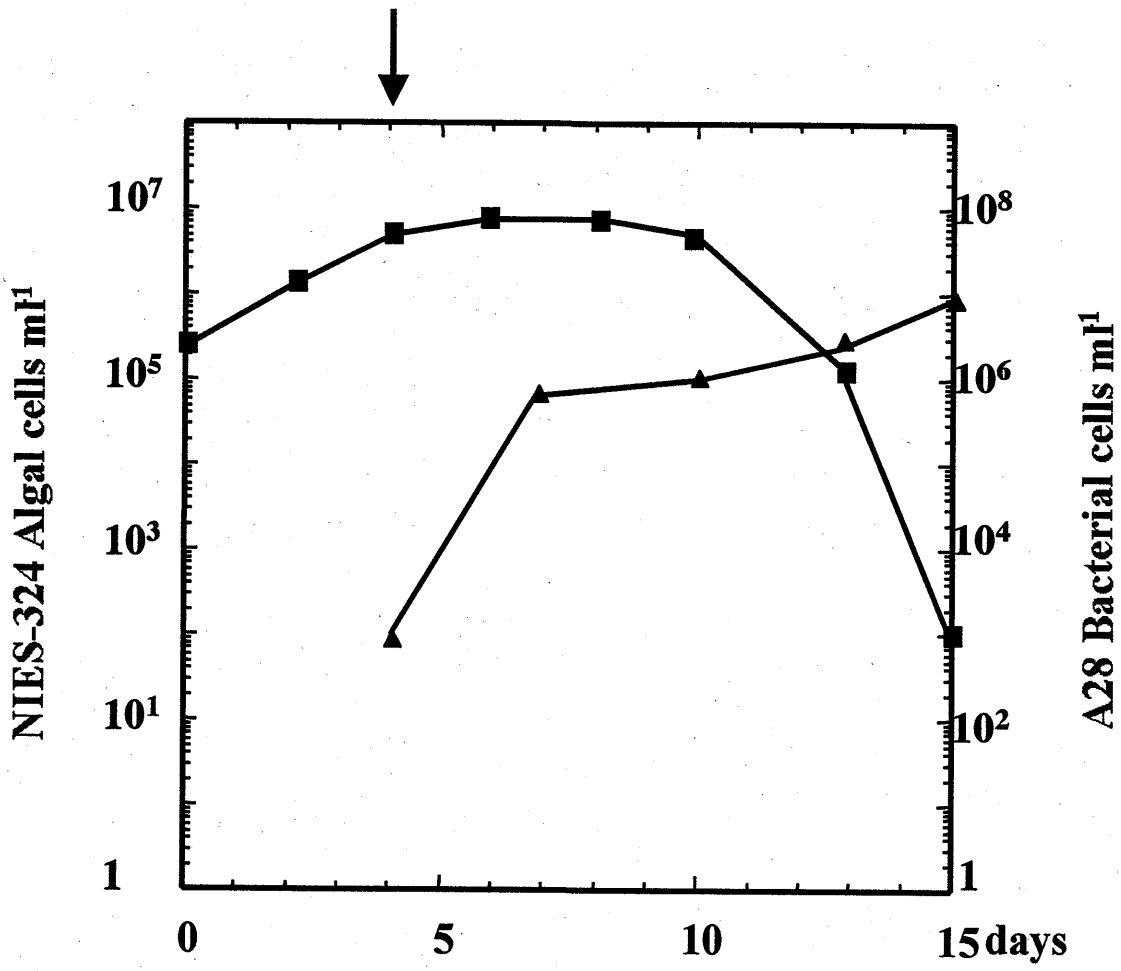

Fig. 2.1. Influence of *Pseudoalteromonas* sp. strains A28 on the growth of *S. costatum* strain NIES-324. *S. costatum* was grown in modified SWM-III medium in the presence of A28. Bacterial cells of A28 were added to *S. costatum* culture 4 days after the start of cultivation, as indicated by the arrows. Symbols:■, algal cells; ▲, bacterial cells.

2.3.2 Algicidal activity of strain A28culture supernatant

The culture supernatant of strain A28 showed potent algicidal activity (Fig. 2.2). When the A28 culture supernatant was applied to a paper disk placed on the lawn of *S. costatum* NIES-324 cells, clear zones were detected around the paper disk (Fig. 2.2, a). No clear zone was detected with fresh ASWM medium. The algicidal activity of the A28 culture supernatant was labile to heating at 100°C for 15 min (Fig. 2.2, b). The culture supernatant was then subjected to ultrafiltration with a 10,000-M_w-cutoff membrane, and the filtrate and concentrated supernatant were examined for the ability to kill *S. costatum* by using the paper disk assay technique. The filtrate failed to form clear zones around paper disks (Fig. 2.2, c). Whereas, concentrated supernatant showed algicidal activity (Fig. 2.2, d). These results suggest that strain A28 produced extracellular substances having algicidal activities.

2.3.3 Isolation of algicidal activity deficient mutants

The mutants lacking algicidal activity were isolated after NTG mutagenesis to investigate detail elucidations of requisite for killing *S. costatum* of the strain A28 culture supernatant. A total of approximately 3,000 clones were examined for the ability to kill *S. costatum* cells, and two mutants, designated NH1 and NH2, were unable to form detectable plaques on the *S. costatum* lawns (Fig. 2.3). It was also confirmed that neither NH1 nor NH2 killed *S. costatum* NIES-324 in mixed algal-bacterial cultures (Fig. 2.4). There were no influences of NH1 and NH2 to *S. costatum* cultivation.

2.3.4 Enzyme assays of culture supernatants of A28 and mutants strain

To identify the extracellular algicidal substances, the concentrated supernatants

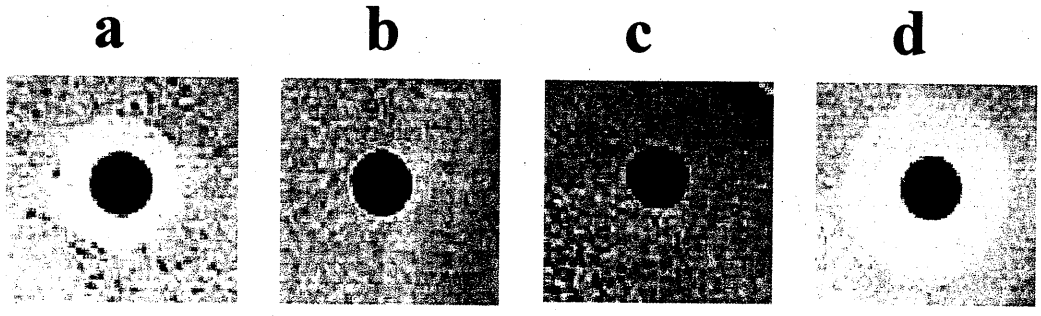

Fig. 2.2. Detection of algicidal activity of culture supernatant of strain A28. Zones of clearing around paper disks indicate lysis of *S. costatum* cells. a : A28 culture supernatant (20 _l), b : heated supernatant (20 _l), c : ultrafiltration of culture supernatant, d : culture supernatant concentrated by ultrafiltration and reconstitued by fresh medium (20 _l)

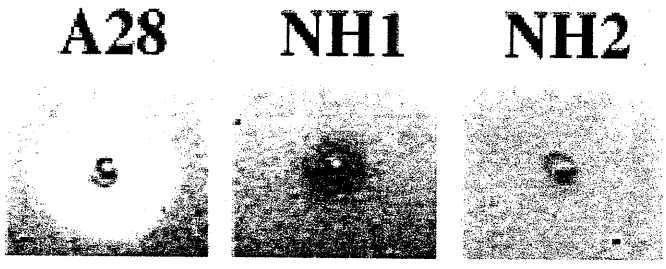

Fig. 2.3. Detection of algicidal activity of strain A28, NH1, and NH2. Zones of clearing around the colonies lysis of *S. costatum* cells.

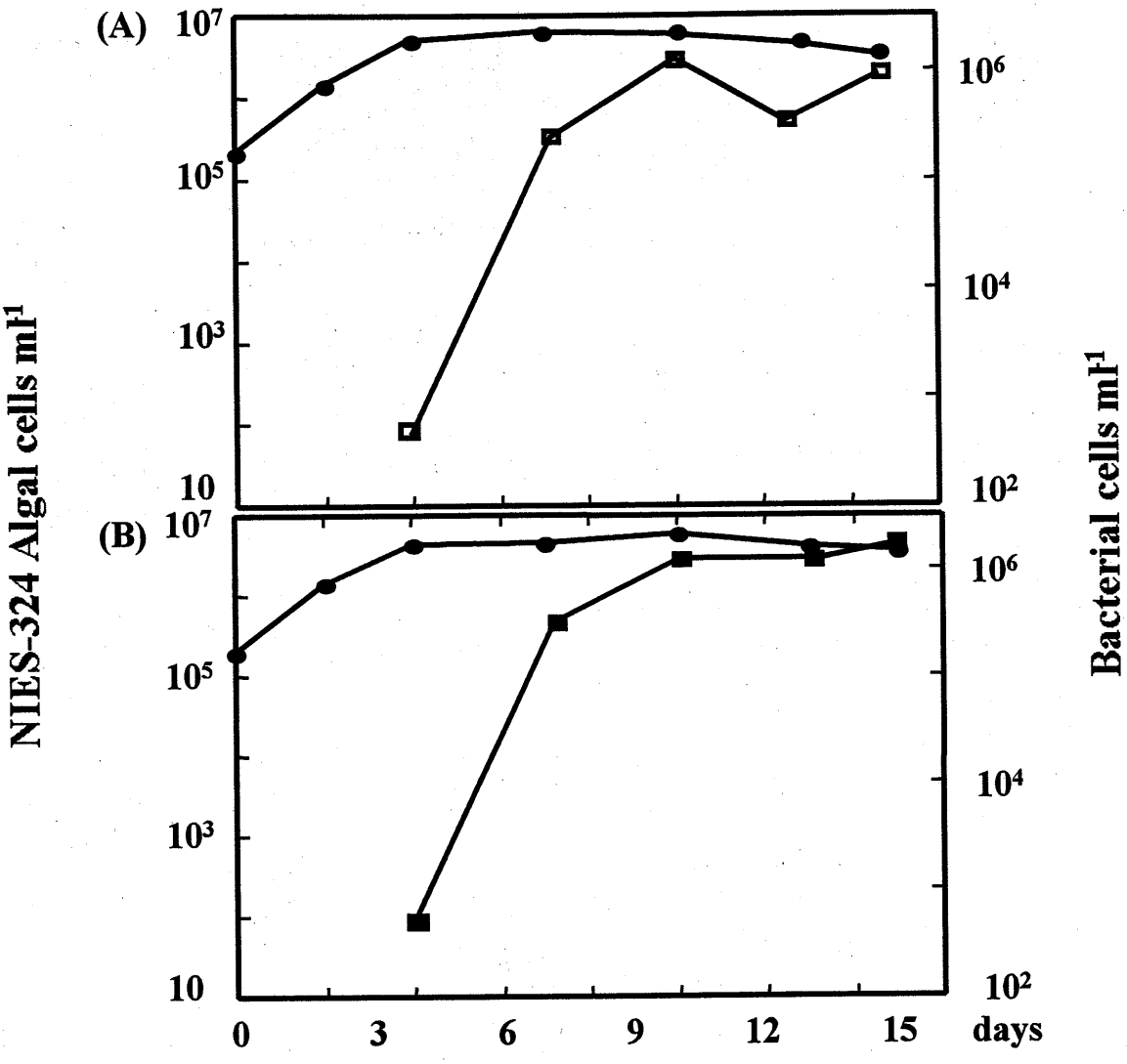

Fig. 2.4. Influence of *Pseudoalteromonas* mutant strains NH1 and NH2 on the growth of *S. costatum* strain NIES-324. *S. costatum* was grown in modified SWM-III medium in the presence of NH1 (A) and NH2 (B). Symbols● , algal cells; ◘ , NH1; ■ , NH2 bacterial cells.

Table 2.1. Enzymatic activities of the culture supernatants of *Pseudoalteromonas* sp. strain A28 and mutants

Strain	Enzymatic activity (U ml of supernatant^{-1})			
	Protease	DNase	Cellulase	Amylase
A28	8.2	9.4	—	—
NH1	0.9	8.8	—	—
NH2	1.1	9.0	—	—

were further examined for their activities of various enzymes, including protease, DNase, cellulase, and amylase. The concentrated A28 supernatants showed protease and DNase activities (Table 2.1), while cellulase and amylase activities were not detected. In addition, the agar plate assay convincingly showed that A28 cells had protease and DNase activities.

The culture supernatant of either NH1 or NH2 showed at most about 13% of the protease activity detected with the parental strain, A28 (Table 2.1). Both NH1 and NH2 had DNase activities comparable to that of the parental strain (Table 2.1). These results suggest that the extracellular protease of strain A28 is responsible for the algicidal effects.

2.4 Conclusions and discussions

The algicidal bacteria kill or lyse the algal cells by direct and/or indirect attacks. Indirect attack, doesn't need the cell-to-cell contact, was considered chemically mediated with the bacteria produced substances. It is well known that gliding algicidal bacteria, including *Cytophaga*, generally attach directly to algal cells and lyse them (24). In contrast, most algicidal bacteria belonging to the genera *Flavobacterium* (14), *Alteromonas* (27), *Pseudoalteromonas* (29), which belong to the γ-proteobacterial group, are thought to kill algal cells by means of algicidal substances.

Pseudoalteromonas sp. strain A28 (formerly named *Alteromonas* sp. strain A28) (18) was fatal to the diatoms *Thalassiosira* and *E. zodiacs* as well as the raphidophycean flagellate *C. antiqua* (29). Strain A28 was isolated by soft-agar overlay technique (55). When the bacterium was co-cultured with diatom *Skeletonema costatum* on the soft agar plate, *S. costatum* cells were lysed and clear zones formed around the colonies of strain A28 on the lawn of *S. costatum* (Fig 2.3. A28). Strain A28 produced some kinds of extracelluar algicidal substances into the

culture and the algicidal activity of culture supernatant was diminished after heating. Algicidal effects of the culture supernatants of strain A28 were also excluded by ultrafiltration with a 10,000-M_w-cutoff membrane. Therefore, this strain is unlikely to excrete low-molecular-weight substances capable of killing *S. costatum.* To clarify the algicidal substances of strain A28 and investigate in details, mutants which lacking algicidal activity were isolated and compared the extracellular enzymatic activities with parental strain A28. Species of *Pseudoalteromonas* are generally found in association with marine eucaryotes and display the anti-bacterial, bacteriolytic, and algicidal activity. While a wide range of inhibitory extracellular agents such as toxins, bacteriolytic substances, and other enzymes are produced, compounds promoting the survival of other marine organisms living in the vicinity of *Pseudoalteromonas* species have also been found (62). In the case of strain A28, the culture supernatant showed protease and DNase activities. The mutants which lacking algicidal activity showed reduced the extracellular protease activities while, DNase activities comparable to that of the parental strain A28. This result suggesting extracellular protease of strain A28 may have concerning to algicidal ability.

Chapter 3.

Purification and Characterization of Algicidal Extracellular Protease of *Pseudoalteromonas* sp. Strain A28

3.1 Introduction

The algicidal marine bacteria can generally be divided into two groups by their algicidal mechanisms. The first group, directly attacks and lyses target algal cells after cell-to-cell attachment, and the second group produces and excretes algicidal substances to kill algal cells. Fukami et al. (14) reported that *Flavobacterium* sp. strain 5N-3 produced a basic compound with a molecular mass of less than 500 Da to kill the dinoflagellate *Gymnodinium nagasakiense*. It has also been reported that *Pseudoalteromonas tunicata* produced an extracellular component with algicidal activity toward to algal spores that is heat-sensitive, polar and between 3 and 10 kDa in molecular size (12). *Pseudomonas* sp. strain T827/2B killed the diatom *Thalassiosira pseudonana* by excreting a heat-labile compound having a relatively high molecular weight (3). While the many algicidal bacteria are thought to produce algicidal substances, most of these substances have not been purified and little is known about the mechanisms of algal lysis at the molecular level.

Pseudoalteromonas sp. strain A28 kills diatom *Skeletonema costatum* by producing extracellular substance. In genetic investigations of strain A28 and algicidal activity deficient mutants, extracellular protease was thought to be concerning the algicidal activity for culture supernatant of strain A28. To make clear whether the extracellular protease of strain A28 has algicidal activity or not,

protease was purified to homogeneity from the concentrated culture supernatant of strain A28 in this chapter. The purified protease has molecular weight of 50 kDa, serine protease, showed algicidal activity to diatom *Skeletonema costatum* NIES-324.

3.2 Materials and methods

3.2.1 Enzyme purification

Pseudoalteromonas sp. strain A28 cultures were grown for 5 h at 28°C in ASWM medium, and the cells were harvested by centrifugation at 6,500 × *g* for 15 min. The culture supernatant was filtered through a 0.45-μm-pore-size nitrocellulose membrane filter (Advantec Inc., Tokyo, Japan) to remove any remaining bacteria. Filtrate of 3 liter was concentrated to approximately 100 ml by using a stirred ultrafiltration cell equipped with a 10,000-M_w-cutoff membrane (Advantec Inc.). The concentrated sample was then dialyzed against 20 mM Tris-HCl buffer (pH 8.0). The dialyzed sample was applied to an anion-exchange column (Poros HQ/M, 4.6 by 100 mm; PerSeptive Biosystems Inc., Framingham, Mass.). The column was washed with 20 mM Tris-HCl buffer (pH 8.0), and proteins were eluted with a linear NaCl gradient of 0 to 1 M in 20 mM Tris-HCl buffer (pH 8.0). Fractions with high protease activities were pooled and stored at -80°C until they were used for preparative native-protein gel electrophoresis. Preparative native-protein gel electrophoresis was performed using a mini-preparative cell (Bio-Rad) (13). The lower gel (4 cm) contained 8% polyacrylamide and 376 mM Tris-HCl (pH 8.8), while the upper one (1.5 cm) contained 4% polyacrylamide and 124 mM Tris-HCl (pH 6.8). The electrode and elution buffers contained 25 mM Tris and 192 mM glycine (pH 8.3). The sample buffer contained 62 mM Tris-HCl (pH 6.8). A 500 μl sample was mixed with 500 μl of 25% (wt/vol) glycerol and 0.012% bromophenol blue stacking dye in 62.5 mM

Tris-HCl (pH 6.8) before being applied to the gel column. Electrophoresis was conducted at 400 V and 3 mA. The elution of protease was complete after 5 h at a flow rate of 0.1 ml min^{-1}. The protein concentration was determined by the bicinchoninic acid method (60). The purity of the protease was determined by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (33). Protein bands were visualized by using the silver stain method (45). The native-protein molecular mass was measured by gel filtration (Superdex 200 column, 1.0 by 30 cm; Pharmacia) with a flow rate of 0.5 ml min^{-1} (with buffer containing 25 mM potassium phosphate–0.1 M NaCl [pH 7.0]). The molecular standard used for gel filtration chromatography included bovine serum albumin (67 kDa), hen egg ovalbumin (43 kDa), bovine pancreas chymotrypsinogen (25 kDa), and bovine pancreas RNase A (14 kDa).

3.2.2 Enzyme assay conditions

The protease activity during purification was measured as azocasein (Sigma) hydrolytic activity as described in chapter 2. One unit of protease activity was defined as the amount of enzyme that caused an incremental change of one absorbance unit per hour. The protease activity was also measured by using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) as a substrate (56). A standard assay mixture contained 2.5 mM succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, 50 mM Tris-HCl (pH 7.8), and 2% dimethylformamide. After 10 min of incubation, the absorbance of the reaction mixture was measured at 410 nm. One unit of the enzyme was expressed as the enzymatic activity giving an absorbance of 1.0 under the above-described conditions.

The pH dependence of activity was determined by using 50 mM acetate buffer at pHs of 4.2 and 5.0, 50 mM phosphate buffer at pHs of 6.0 and 7.0, and 50 mM Tris buffer at pHs of 7.8, 8.8, and 9.3. Protease activity was measured at 30 °C. For,

thermostability test, the purified protein was pre-incubated at 4, 20, 30, 37, 42, 50, and 60 °C for 30 min in 50 mM Tris-HCl (pH 7.8) buffer then protease activity was measured at 30 °C.

The algicidal activity of purified protease is performed by the method of paper disk assay described as chapter 2. Algicidal activity was assessed by the presence of clear zones around the paper disks.

To investigate *S. costatum* cells digestion by the purified protease, cell free extract of *S. costatum* was prepared. *S. costatum* cells were harvested by centrifugation at 10,000 × *g* for 10 min then resuspended by 10mM Tris buffer (pH 8.0) and lysed by sonication with five 20-s pulses generated by a Sonifier (Branson, Danbury, Conn.). After each 20-s burst, the cells were cooled for 5 min in a wet-ice bath. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was incubated with adding purified protease at 30°C. The reaction was stopped by adding PMSF to final concentration 1 mM.

3.2.3 Inhibitor studies

Protease inhibitors tested in this study were phenylmethylsulfonyl fluoride (PMSF; Sigma), diisopropyl fluorophosphate (DFP; Katayama, Osaka, Japan), leupeptin (Nacalai Tesque, Inc., Kyoto, Japan), antipain (Nacalai Tesque, Inc.), chymostatin (Sigma), pepstatin (Nacalai Tesque, Inc.), *1,10*-phenanthroline (Sigma), tetraethylenepentamine (Sigma), EDTA (Sigma), and EGTA (Sigma). The mixture of each protease inhibitor and enzyme solution appropriately diluted in 50 mM Tris-HCl (pH 7.8) was incubated at room temperature for 30 min before succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was added. Protease activity was measured at 30°C by the method described above.

3.2.4 Amino acid sequence analysis

To determine the N-terminal amino acid sequence, the purified protease was subjected to SDS–12.5% PAGE and then electroblotted onto an Immobilon-P membrane (Millipore Corporation, Bedford, Mass.), and stained with Coomassie brilliant blue. The protein band was excised and N-terminal amino acid sequence was determined with a Procise protein sequencing system (Applied Biosystems, Foster City, Calif). Internal amino acid sequences of trypsin-digested peptide of purified protease were determined by mass spectrometry. An amino acid sequence similarity search was done with the FASTA program (48) with the Protein Identification Resource amino acid sequence database.

3.3 Results

3.3.1 Protease purification

Protease was purified to homogeneity from the concentrated culture supernatant of strain A28 by ion-exchange chromatography, followed by preparative gel electrophoresis. The purified protease designated as AspI. The results of a typical enzyme purification procedure are summarized in Table 3.1. Chromatography of the concentrated culture supernatant on a Poros HQ/M anion-exchange column resolved two peaks of protease activity (Fig. 3.1). The two peaks of activity were eluted with approximately 300 and 500 mM NaCl, respectively. However, paper disk assays revealed that only the first peak fraction had algicidal activity. The proteases, which were detected in the first and second peak fractions, were designated AspI and AspII, respectively. The fractions of AspI showing algicidal activity were further purified by preparative native-protein gel electrophoresis. After preparative native-protein gel electrophoresis, SDS-PAGE analysis showed a single protein band (Fig. 3.2). When protease AspI was applied to a paper disk placed on the lawn of *S. costatum* NIES-324 cells, clear zones were detected around

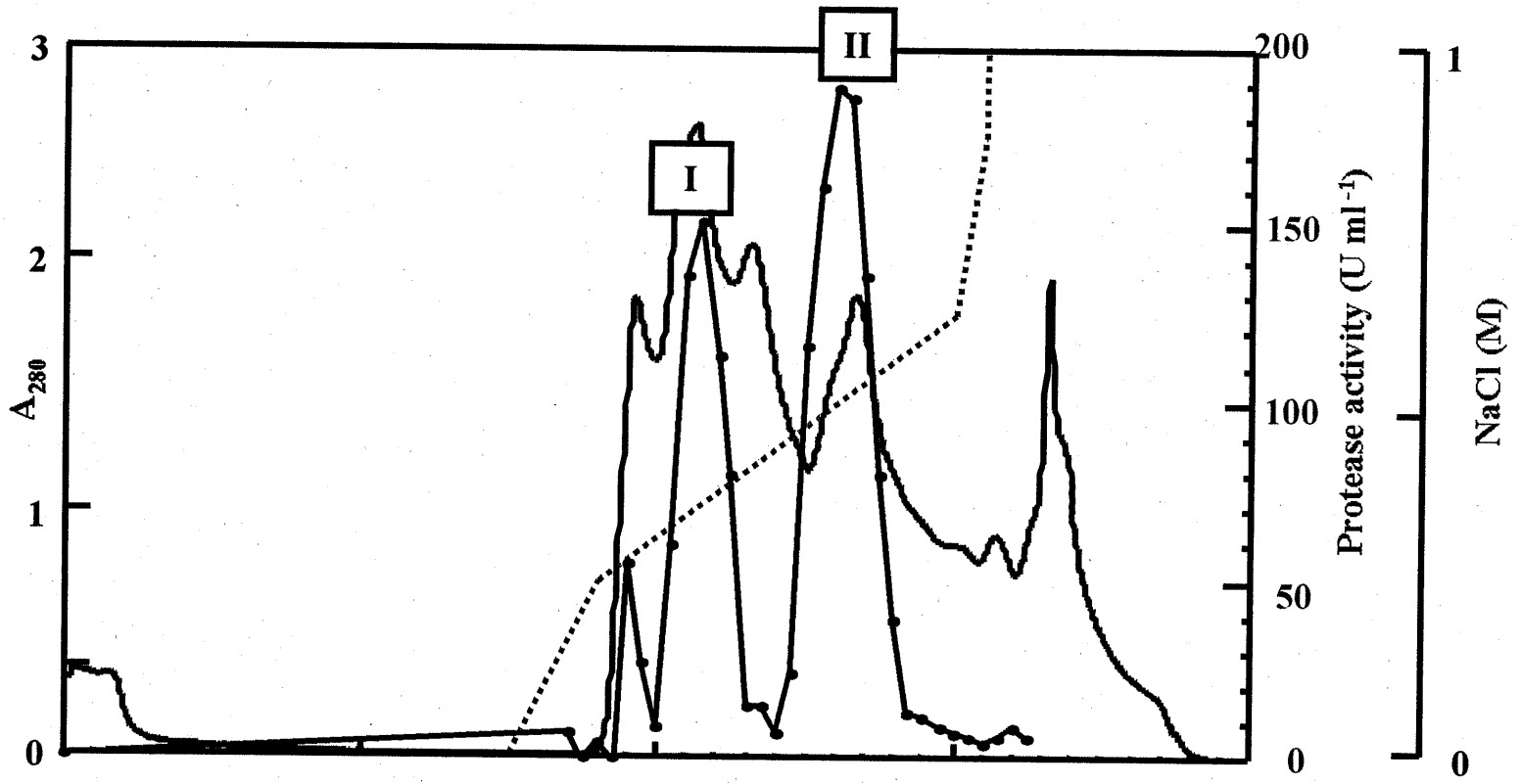

Fig. 3.1. Elution profiles of the protease activities from a Poros HQ/M anion-exchange column. The concentrated culture supernatants of *Pseudoalteromonas* sp. strain A28 was applied to column. The column was developed with a linear NaCl gradient of 0 to 1 M in 10 mM Tris-HCl buffer (pH 8.0). —, absorbance at 280nm; —●—, protease activity (U ml^{-1}); ·····, concentraion of NaCl (M)

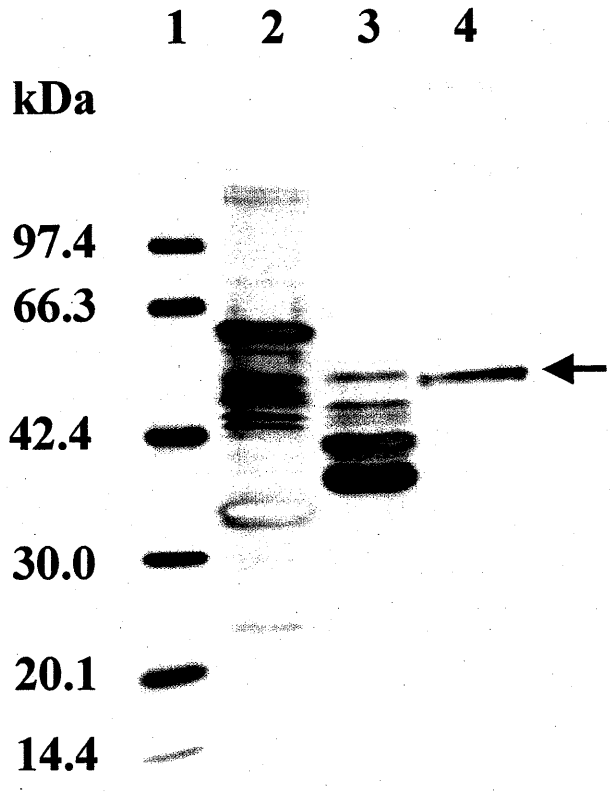

Fig. 3.2. SDS-PAGE analysis of AspI-enriched fractions obtained during enzyme purification. The pooled samples from each of the purification steps were subjected to electrophoresis and silver stained. Lane 1, molecular mass markers; lane 2, concentrated culture supernatant (20 _g); lane 3, AspI-rich fraction from anion-exchange chromatography (10 _g); and lane 4, AspI-rich fraction from preparative native-protein gel electrophoresis (3 _g).

Table 3.2. Effects of inhibitors on protease activity of AspI

Protease inhibitor	Specificity(ies) of inhibitor	Inhibitor conc.	Relative activity (%)
None			100
Phenylmethylsulfonyl fluoride	Serine	1mM	0
Diisoprophyl fluorophosphate	Serine	1mM	5
Leupeptin	Ser/Cys	1mM	1
Pepstatin	Aspartic	1mM	72
EDTA	Metallo	1mM	94
EGTA	Metallo	1mM	77
1, 10-phenanthroline	Metallo	1mM	87
Tetraethylenepentamine	Metallo	1mM	83
Chymostatin	Chymotrypsin	50ppm	3
Antipain	Papain, trypsin, cathepsin A,B	0.1mM	4

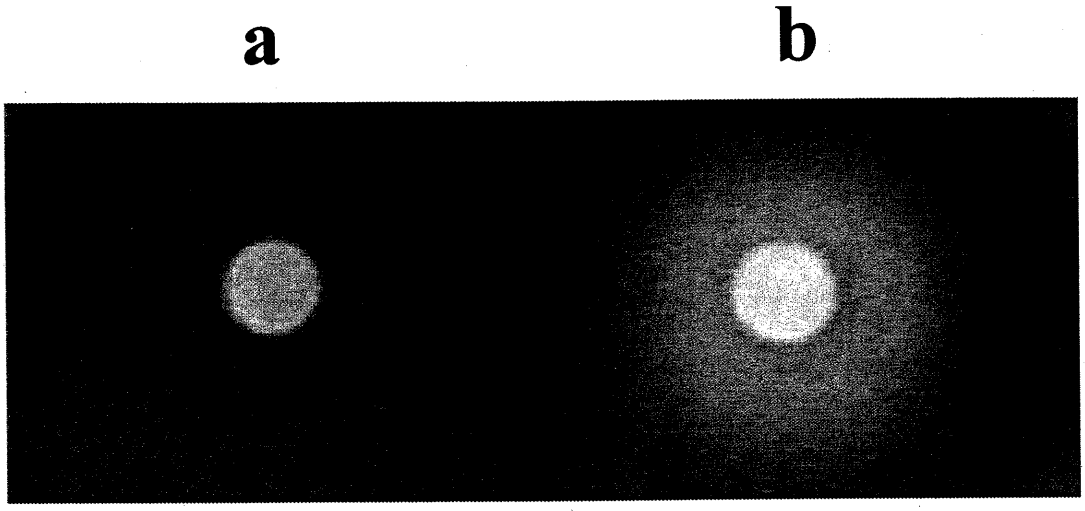

Fig. 3.3. Detection of algicidal activity of purified extracellular protease of *Pseudoalteromonas* sp. strain A28. Algicidal activity was detected by formation of clear zone on the *S. costatum* lawn. a, preparative native electrpphoresis buffer as control (20 _l); b, purified AspI (20 _l: 0.16U of protease activity).

the paper disk (Fig. 3.3). Agar blocks in the clear zones were then cut out and inoculated into fresh modified SWM-III medium. No growth or *S. costatum* was observed, confirming that AspI is algicidal substance.

3.3.2 Enzyme properties

By means of SDS-PAGE, the molecular mass of AspI was estimated to be 50 kDa. Since the molecular mass of AspI was also estimated to be 50 kDa by gel filtration, AspI should be a monomer. AspI was able to cleave succinyl-Ala-Ala-Pro-Phe-_-nitroanilide. However, neither succinyl-Ala-Ala-Val-Ala-_-nitroanilide nor tosyl-Gly-Pro-Lys-_-nitroanilide was cleaved by AspI. Effects of temperature and pHs on the activity of purified protease was studied with succinyl-Ala- Ala-Pro-Phe-*p*-nitroanilide as substrate. The optimum temperature for hydrolysis activity was 30 °C. To examine the heat stability, the enzyme solution was allowed

to stand 30min at various temperatures, and then the residual activity measured. As shown in Fig. 3.4A, protease activity maintained only 10 % of its activity at 60 °C. The protease activity was abolished by incubation at 68 °C for 1 h or 100°C for 15 min. The optimum pH was 8.8. And 4, 5, 13, 81, and 61% of enzyme activity corresponding to pH 8.8 were detected at pHs 4.2, 5.0, 6.0, 7.0, and 9.3, respectively (Fig. 3.4 B). The pH stability was determined by the measurement of the residual activity at pH 7.8 after incubation at various pHs at 20 °C for 30min. Protease activity remained over 90 % of its activity at pH 6.0, 7.0, 8.8, and 9.3.

The AspI and *S. costatum* cell free extract were mixed and reacted at 30°C then digested cell free extract analysis by SDS-PAGE electrophoresis. The digested protein band of *S. costatum* cell free extract was excreted then internal amino acid sequence were determined by mass spectrometry. The determined amino acid sequence was Asp-Asn-Asp-Gln-Asn-Ser-Leu-Asn, which showed similarity with outer membrane precursor of the bacterium *Treponema pallidum.*

Ten enzyme inhibitors were tested for the ability to block the hydrolysis of succinyl-Ala-Ala-Pro-Phe-ρ-nitroanilide (Table. 3.2). PMSF (1 mM), DFP (1 mM), and chymostatin (0.1 mM), which are inhibitors of serine proteases, completely inhibited the activity of AspI. Antipain (0.1 mM) and leupeptin (1 mM), which inhibit both serine and cysteine proteases, also caused complete inhibition of AspI activity. No significant inhibition was detected with the metal protease inhibitors, including EDTA (1 mM), EGTA (1 mM), *1,10*-phenanthroline (1 mM), and tetraethylenepentamine (1 mM). Pepstatin (1 mM), an inhibitor of aspartic protease, did not inhibit the activity of AspI.

The N-terminal amino acid sequence of purified AspI was determined to be Ala-Thr-Pro-Asn-Asp-Pro. Only six N-terminal amino acids could be determined because cleavage of the peptide bonds after Pro proceeded very slowly (Procise protein sequencing system user's manual, Applied Biosystems). Internal amino acid sequences were also determined to be Gly-The-Gly-Asp-Ala-Asp-Leu-Tyr-Val-

Table 3.2. Effects of inhibitors on protease activity of AspI

Protease inhibitor	Specificity(ies) of inhibitor	Inhibitor conc.	Relative activity (%)
None			100
Phenylmethylsulfonyl fluoride	Serine	1mM	0
Diisoprophyl fluorophosphate	Serine	1mM	5
Leupeptin	Ser/Cys	1mM	1
Pepstatin	Aspartic	1mM	72
EDTA	Metallo	1mM	94
EGTA	Metallo	1mM	77
1, 10-phenanthroline	Metallo	1mM	87
Tetraethylenepentamine	Metallo	1mM	83
Chymostatin	Chymotrypsin	50ppm	3
Antipain	Papain, trypsin, cathepsin A,B	0.1mM	4

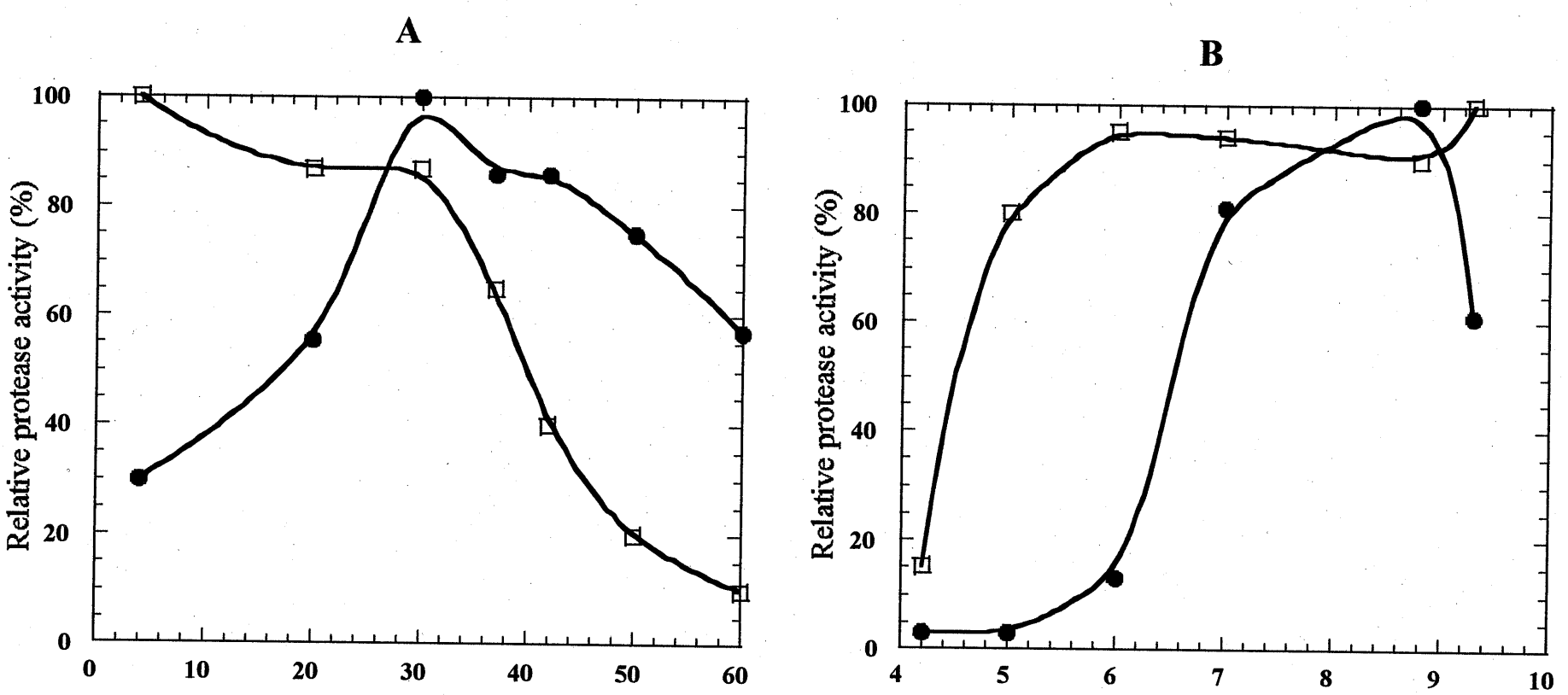

Fig. 3.4. Effects of temperature and pH on the activity and stability of AspI protease activity. (A) Protease actives were measured at various temperature and stabilities were measured by pre-incubating at above temperature and remaining activities were measured. (B) Protease activities were measured at various pHs, and pre-incubated on above pHs and remaining activities were measured. —●— ; activities, —□— ; stabilities

Pro, and Ser-Ser-Thr-Thr-Gln-Ser-Ala-Leu-Asn. A computer-assisted similarity search revealed that the N-terminal amino acid sequence of AspI was identical to that of the mature alkaline serine protease of *Alteromonas* sp. strain O-7 and high similarity with internal amino acid sequences (68).

3.4 Conclusion and Discussions

In this chapter showed that a serine protease, designated AspI, was responsible for the algicidal effects. The algicidal effects of the culture supernatants were excluded by ultrafiltration with a 10,000-M_w -cutoff membrane. Strain A28 is unlikely to excrete low-molecular-weight substances capable of killing *S. costatum.* Inhibition studies revealed that DFP and PMSF abolished AspI activity. Both DFP and PMSF are known to be serine protease inhibitors, which irreversibly react with the active-site serine residues (10, 19). Antipain and leupeptin also caused complete inhibition of AspI activity. Although antipain and leupeptin are cysteine protease inhibitors, they are also known to inhibit the serine protease trypsin (2, 65). These results suggest that AspI is a serine protease. The N-terminal amino acid sequence of AspI was identical to that of the alkaline serine protease (AprI, class I subtilase) of *Alteromonas* sp. strain O-7 (59, 68). *Alteromonas* sp. strain O-7 was isolated from a sediment sample at the Sagami Bay of Japan as a chitin-degrading bacterium (69). The molecular mass of the mature AprI (35 kDa) is smaller than that of AspI (50 kDa). It is not known whether AprI has potent algicidal activity. Strain A28 produced two proteases, AspI and AspII. AspII was sensitive to PMSF, indicating that it was also a serine protease. SDS-PAGE of purified protease II revealed two protein bands corresponding to molecular masses of 48 and 33 kDa. However, non-denaturing PAGE of protease II showed a single band at 75 kDa. These results indicate that AspII is a heterodimer. Unlike AspI, AspII did not show any algicidal activity. This may suggest that AspII had substrate specificities

different from those of AspI. When bovine serum albumin was degraded by AspI and AspII, the patterns of cutting were not identical. Alternatively, they may have different affinities for *S. costatum* cells. The affinity of the protease for algal cells should be of importance for causing the algicidal effects. Some commercially available proteases, including trypsin, pepsin, subtilisin, and pronase, were examined for their algicidal activities, but none of them showed algicidal activity.

Chapter 4.

Cloning and Characterizations of Algicidal Substance Gene from *Pseudoalteromonas* sp. Strain A28 Chromosomal DNA

4.1 Introduction

One of the extracellular protease of *Pseudoalteromonas* sp. strain A28, AspI, had algicidal activity. AspI was a monomeric protein with a molecular mass of 50kDa. Protease activity of AspI was strongly inhibited by PMSF, which is serine protease inhibitor. Another extracellular serine protease of strain A28, AspII was also purified but no algicidal activity detected. This led to study, which describes the molecular cloning and nucleotide sequence of the AspI gene. Thereby enabling elucidation of its structure and function.

In this chapter describes cloning of the gene for the algicidal substance, AspI. The construction and complementation of AspI-lacking mutant strain of strain A28 are described.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

Pseudoalteromonas sp. strain A28 is an algicidal bacterium isolated from the Ariake Sea of Japan. A28 was grown at 28 °C with shaking in ASWM medium.

Escherichia coli MV1184 was used for plasmid construction and DNA manipulation and the cells were grown at 37 °C in Luria-Bertani (LB) medium (40). Whenever necessary, ampicillin and kanamycin were added at final concentrations of 100 and 200μgml^{-1}, respectively. For electroporation of strain A28, LBN (containing 24g NaCl per liter 1mM $MgCl_2$, and 300 μM $CaCl_2$) medium was used (66).

4.2.2 Cloning and nucleotide sequencing of *aspI* gene from chromosomal DNA of strain A28

A computer-assisted similarity search revealed that the N-terminal amino acid sequence of AspI was identical to that of the mature alkaline serine protease (AprI-M) of *Alteromonas* sp. strain O-7 and determined internal amino acid sequences of purified AspI were showed high similarities to those of AprI-M (68). Therefore, for the cloning of strain A28 *aspI* gene, AprI-M (mature region of AprI) gene encoding DNA fragment was used as probe of A28 chromosomal DNA Southern hybridization. Genomic DNA of strain A28 was digested with various restriction enzymes and electrophoresed on 1% agarose gel, then Southern hybridization was performed by standard procedures (61). Positive DNA fragment in the southern hybridization was cloned by a standard method (51) using pUC118 (70) and pBluescriptII KS+. *E. coli* cells were cultivated at 37°C in LB medium. When necessary, ampicillin (50 mg ml $^{-1}$) was added to the medium. The nucleotide was sequenced with double-stranded plasmid DNA as a template by the dideoxynucleotide chain termination methods (52) with the Dye Terminator Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems, Inc.). Nucleotide and deduced amino acid sequences were analyzed and compared with other known sequences in the GenBank library of DNA sequences by use of DNASIS programs (Hitachi Software Engineering, Kanagawa, Japan) and the FASTA program (48).

4.2.3 Construction of a AspI-inactivated mutant of strain A28

Transposon mutagenesis of strain A28 *aspI* gene was carried out using the kanamycin resistance gene (Km^r) cassette from pUC4K (71). pBSPKm was constructed as follows (Fig. 4.1). The resultant plasmid, pBSPKm, was transformed into strain A28 by electrophoresis. Cells grown overnight in LBN medium were inoculated into fresh LBN medium (a 1% inoculum), and after 4 h of incubation, cells were harvested by centrifugation (10,000 × *g*, 5min, 4℃). Pellets were resuspended in ice-cold HS buffer containing 7mM HEPES and 232mM sucrose (pH 7.0), washed three times with the same buffer, and resuspended in 400 μl of bacterial suspension was used for electroporation. Electroporation was performed with Electro Cell Manipulator 630 (BTX Inc., San Diego, Calif.) at a capacitance of 50 μF and a voltage of 2.2kV. After electroporation, cells were allowed to grown ASWMglt (ASWM containing 20mM Glutamic acid) medium overnight and spread on LBN agar plate supplemented with 200 μg of kanamycin per ml to obtain *aspI* gene transposon mutant. Chromosomal DNA from Km^r mutants of strain A28 was isolated and Southern hybridization was carried out by standard methods using the kanamycin cassette and *Hin*dIII 2.1kb fragment of strain A28 AspI gene as probes. Algicidal activity and protease activity of culture supernatant of AspI-inactivated mutants were measured by paper disk assay and azocasein hydrolytic activity as described in chapter 2.

4.3 Results

4.3.1 Molecular cloning of the *Pseudoalteromonas* sp. strain A28 *aspI* gene

For the cloning of *aspI* gene of strain A28, extracellular alkaline serine protease

AprI that produced by the marine bacterium *Alteromonas* sp. strain O-7 encoding gene fragment was used as probe. A computer-assisted similarity search revealed that the N-terminal amino acid sequence of AspI was identical to that of the mature alkaline serine protease (AprI-M) of *Alteromonas* sp. strain O-7 and internal amino acid sequences were showed high similarities to those of AprI. In Southern hybridization analysis this probe strongly hybridized with either a 2 to 2.3-kb *Eco*RI fragments or *Hin*dIII fragments when the genomic DNA of strain A28 was completely digested with *Eco*RI or *Hni*dIII. The 2 to 2.3-kb *Eco*RI fragments were cloned into the *Eco*RI site of pBluescriptII KS+ and constructed pPASP0.1. Preliminary DNA sequencing of the plasmid pASP0.1 was indicated that this insert fragment encoded the part of the open reading frame of AspI including the determined N-terminal amino acid sequence of purified AspI. To isolate the remaining part, the 2.1-kb *Hin*dIII fragment of the chromosomal DNA was cloned into pBluescript KS+ to construct pASP0.2 and insert fragment was sequenced. The pPASP0.2 insert overlapped with the pPASP0.1 insert by a 0.8-kb *Eco*RI-*Hin*dIII fragment. Thus, two plasmids were recombined into plasmid pBluescript KS+ to construct pPASP1.0 which sequence covering the entire coding region of AspI (Fig. 4. 2).

4.3.2 Nucleotide sequence of *aspI* gene

The nucleotide sequence of the 3.3-kb insert revealed one major open reading frame (32) of 2,073 bp with the capacity to encode a polypeptide of 691 amino acids and with a molecular size of 71,007 (Fig. 4.3). The N-terminal amino acids of the purified AspI, previously determined by protein sequencing at Ala-149 and showed perfect agreement with the deduced amino acid sequence (Fig. 4.3), suggesting subsequent processing at N-terminus. And also, internal amino acid sequences,

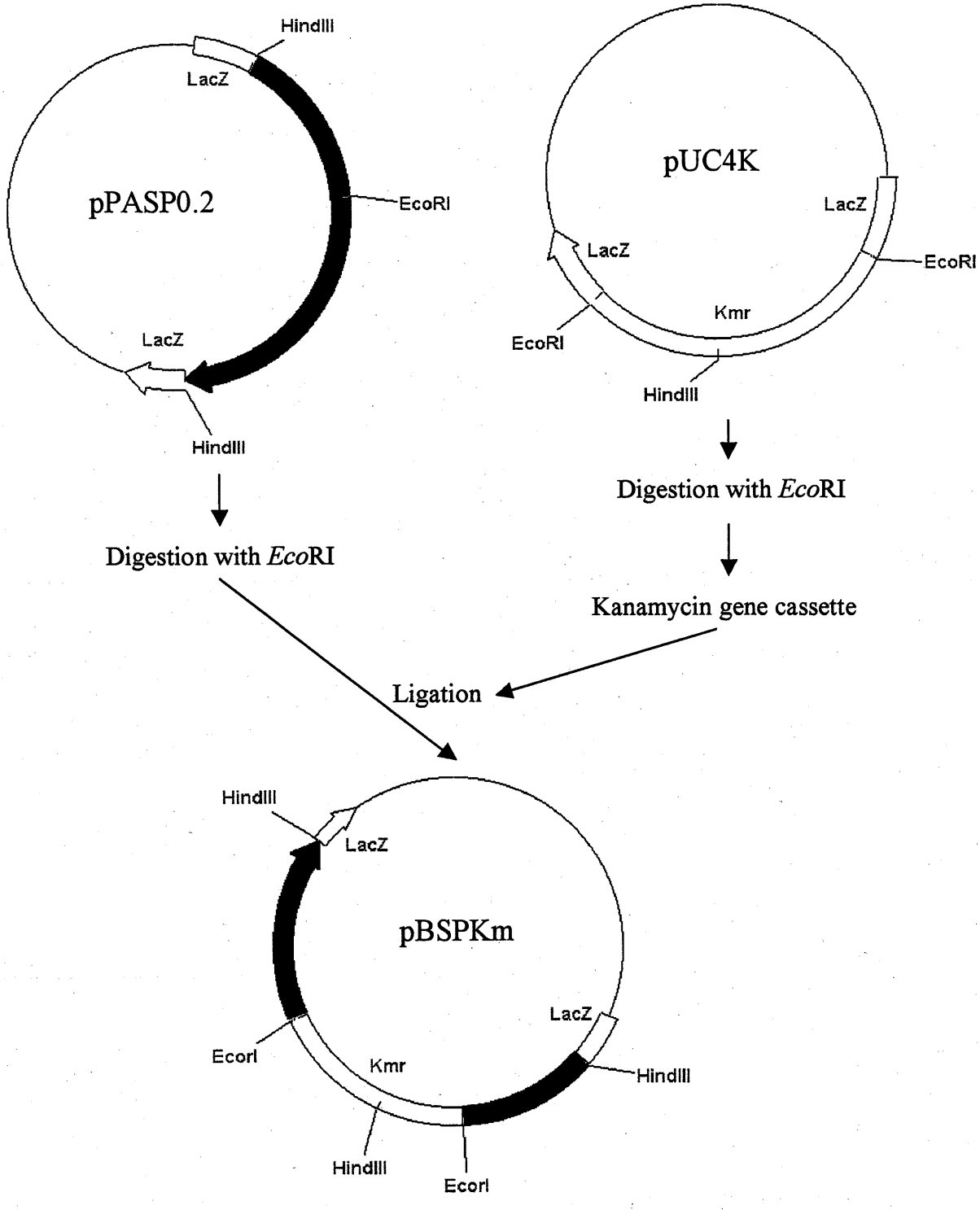

Fig. 4.1. Plasmid construction of Kanamycin cassette insertion on the *aspI* gene. In pPASP0.2, black arrow represents cloned *aspI* gene *Hin*dIII 2.1-kb fragment. In pUC4K, white box represents Km^r cassette. *Eco*RI 1.2-kb fragment of Km^r gene cassette excised from pUC4K was inserted into *Eco*RI site of pPASP0.2 to construct pBSPKm.

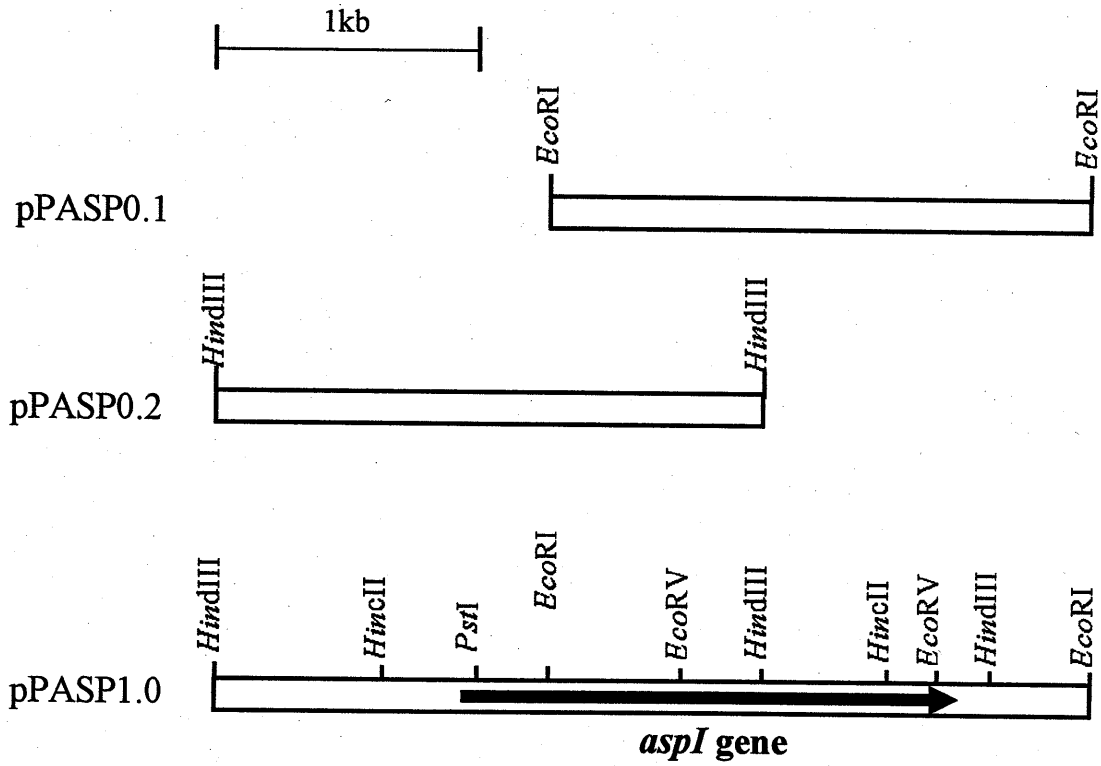

Fig. 4.2. Plasmid construction for cloning and sequencing *aspI* gene

which were determined by mass spectrograph of trypsin-digested peptides of purified AspI, were found starting at Ser-328 (12 amino acids) and at Gly-536 (21 amino acids). This deduced AspI (starting at Ala-149) consist of 543 amino acids and is calculated to be 54.5kDa, which is larger than the 50kDa of the purified AspI suggesting processing at C-terminus.

4.3.3 Deduced amino acid sequence analysis

The amino acid sequence of the *aspI* gene was compared with sequences of other proteins. The overall deduced amino acid sequence of AspI exhibited high levels of sequences similarity to alkaline serine protease of *Alteromonas* sp. strain O-7 (68) with 74.556% identity, extracellular protease of *Xanthomonas campestris* pathovar *campestris* (37) with 52.834% identity, serine metalloprotease of *Burkholderia pseudomallei* (35) with 45.020% identity, and extracelluar serine protease of *Dichelobacter nodosus* (36) with 40.516% identity of amino acid residues which were the members of the subtilase family of serine proteases. The deduced amino acid sequence of AspI was shown high sequence similarity with AprI. The deduced amino acid sequence of AspI was compared with AprI (Fig. 4.4). The predicted product of AspI had 82.9% and 71.5% identity to the mature product (35kDa) and C-terminal pro-region (23kDa) of AprI, respectively (Fig. 4.5). The active sites of AspI were identical with those of serine protease AprI (53, 54). Two repeated amino acid sequences were found in the C-terminal region of AspI. While, the same repeated sequences were in the C-terminal pro-region of AprI. The mature protein of AspI was predicted to contain one of the repeated sequences due to molecular weight of purified AspI.

4.3.4 Characterization of AspI-inactivated mutants of strain A28

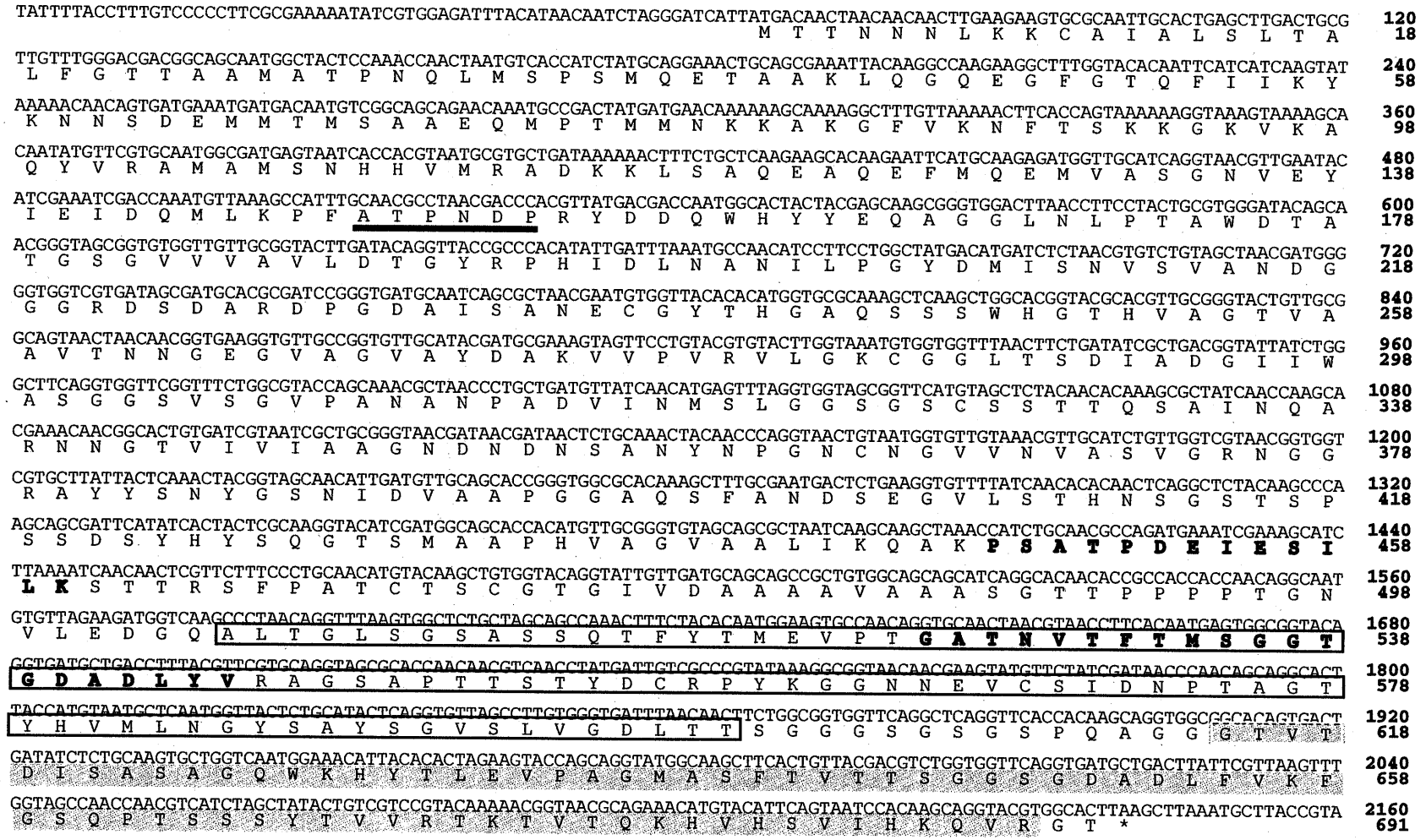

Fig. 4.3. Nucleotide sequence of the *apsI* gene and adjacent DNA regions. The deduced amino acid sequence of AspI is shown below the nucleotide sequence. The stop codon indicated by an asterisk. The N-terminal amino acid sequence of AspI is underlined. The determined internal amino acid sequences were in bold characters. The repeated amino acid sequence of C-terminal region are illustrated solid box and shaded box, respectively.

```
AspI     ATPNDPRYDDQWHYYEQAGGLNLPTAWDTATGSGVVVAVLDTGYRPHIDLNANILPGYDM  208
AprI-M   ATPNDPRYNDQWHYYEAAAGINAPAAWDKATGQGVVVAVLDTGYRPHLDLDANILPGYDM  210
         ******** ******* * * * * *** *** ************** ** *********

AspI     ISNVSVANDGGGRDSDARDPGDAISANECGYTHG-------AQSSSWHGTHVAGTVAAVT  261
AprI-M   ISNTFVANDGGARDNDARDPGDAVTRGECGTDSSGQPVPRADQDSSWHGTHVAGTVAAVT  270
         ***  ****** ** ********    ***            * ****************

AspI     NNGEGVAGVAYDAKVVPVRVLGKCGGLTSDIADGIIWASGGSVSGVPANANPADVINMSL  321
AprI-M   NNGEGVAGVAYDAKVVPVRVLGKCGGLTSDIADGIIWASGGSDR-VPANANPAVVINMSL  329
         ****************************************   ******** ******

AspI     GGSGSCSSTTQSAINQARNNGTVIVIAAGNDNDNSANYNPGNCNGVVNVASVGRNGGRAY  381
AprI-M   GGGGACSATTQNAINQARNNGTVIVIAAGNDNDNSANYNPGNCNGVVNVASVGRDGSRAY  389
         ** * ** *** ****************************************** * ***

AspI     YSNYGSNIDVAAPGGAQSFANDSEGVLSTHNSGSTSPSSDSYHYSQGTSMAAPHVAGVAA  341
AprI-M   YSNYGANIDVAAPGGAQSFADDPEGILSTHNSGSGAPSNDSYHYSQGTSMAAPHVAGVAA  449
         ***** ************** * ** ********  ** **********************

AspI     LIKQAKPSATPDEIESILKSTTRSFPATCTSCGTGIVDAAAAVAAASGTTPPPPTGNVLE  401
AprI-M   LIKQAKPSATPDEVETILKNTTRSFAGSCSNCGTGVVDAAAAVNEALGDVVTPPTGNTLE  509
         ************* * *** *****   *  **** *******  * *    ***** **
```

Fig. 4.4. The deduced amino acid sequence of AspI was compared with AprI (protease from *Alteromonas* sp. O-7). The active sites of serine protease are in bold characters. Identical amino acid residues between AspI and AprI-M (mature protein) are marked by asterisks.

To create a mutant strain deficient in AspI, the clone containing the N-terminal segment of the *aspI* gene on a 2.1-kb *Hin*dIII fragment was mutagenized with the kanamycin resistance gene and introduced into strain A28. The kanamycin resistance (Km^r) gene 1.2-kb *Eco*RI restriction fragment from pUC4k was isolated and cloned into the *Eco*RI site of pASP0.2. This Km^r Ap^r plasmid (pBSPKm) was purified from *E. coli* and electroporated into strain A28. The mutant strain, named SP1, which showed same growth rate with parental strain, was selected on the agar plate containing kanamycin and Southern blot analysis of the genomic DNAs of wild-type strain A28 and SP1 was performed to confirm the Km^r gene integrated into the chromosome by homologous recombination between the AspI sequences on the plasmid and the chromosome (Fig. 4.6B). It is evident from Fig 4.5A that a 2.1-kb chromosomal DNA fragment reacted with the probe, which is N-terminal segment of the *aspI* gene on a 2.1-kb *Hin*dIII fragment in wild-type strain A28. The fragments of 2.0 and 1.3-kb reacted with the same probe in the digested DNAs of mutant SP1 instead of 2.1-kb fragment observed with wild-type strain A28 (Fig. 4.6A panel a). When hybridized performed using kanamycin resistance gene cassette as probe, only the mutant strain SP1 showed signal at 2.0 and 1.3-kb (Fig. 4. 6A panel b) and genomic DNA of strain A28 did not react with this probe. The restriction map of *aspI* gene region on strain SP1 was showed in Fig. 4. 5B.

The mutant strain SP1 reduced extracellular protease activity compared with the parental strain A28 (Fig. 4.7). Strain A28 and SP1 cultures were grown for 18 h at 28°C in ASWM medium then culture supernatant used for assay. The culture supernatant of SP1 was shown only 37.5% protease activity of strain A28 culture supernatant. However this strain still had extracellular protease activity. Strain A28 and SP1 cultures, which sterilized by filtering, added to 6-mm-diameter paper disks on *S. costatum* lawn. *S. costatum* plates were incubated overnight and algicidal activities were judged by forming clear zones around paper disks (Fig. 4. 8). The 12 and 8 mm diameter clear zones were visualized where strain A28 and SP1

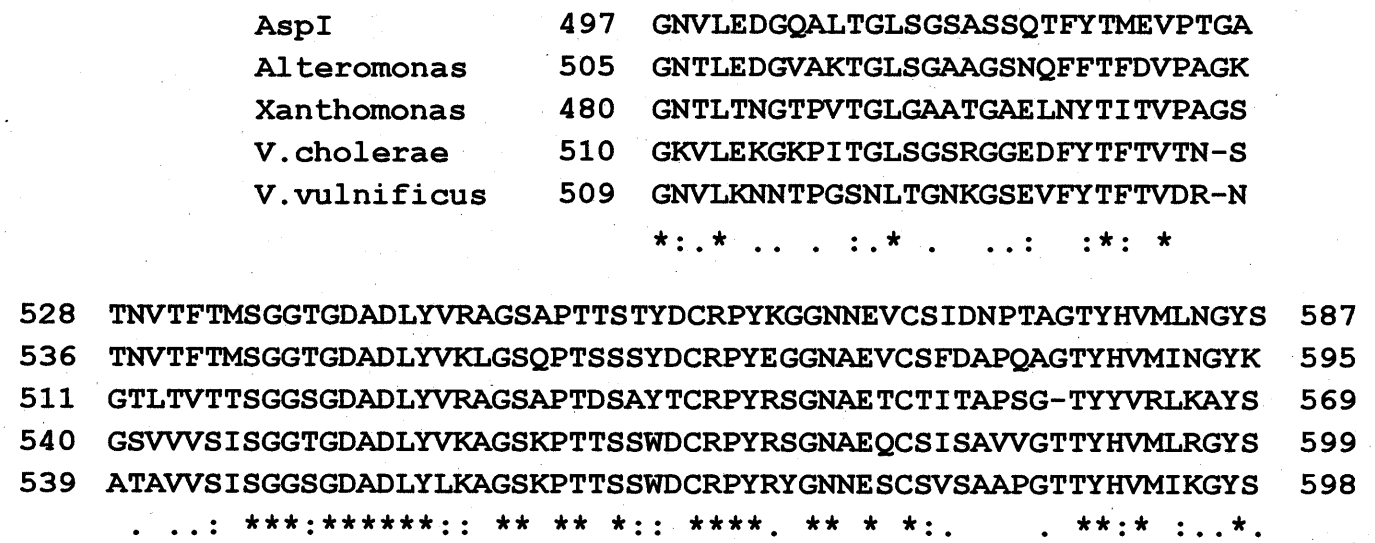

Fig. 4.5. Comparison of the amino acid sequence of the C-terminal regions of AspI with C-terminal pro-region of other proteases. Alteromonas (protease from *Alteromonas* sp. serine protease) , Xanthomonas (protease from *Xanthomonas campestris*), V. choleare (HA/protease from *Vibrio cholerae*), V. vulnificus (metalloprotesas from *Vibrio vulnificus*). Identical amino acid residues are marked by asterisks.

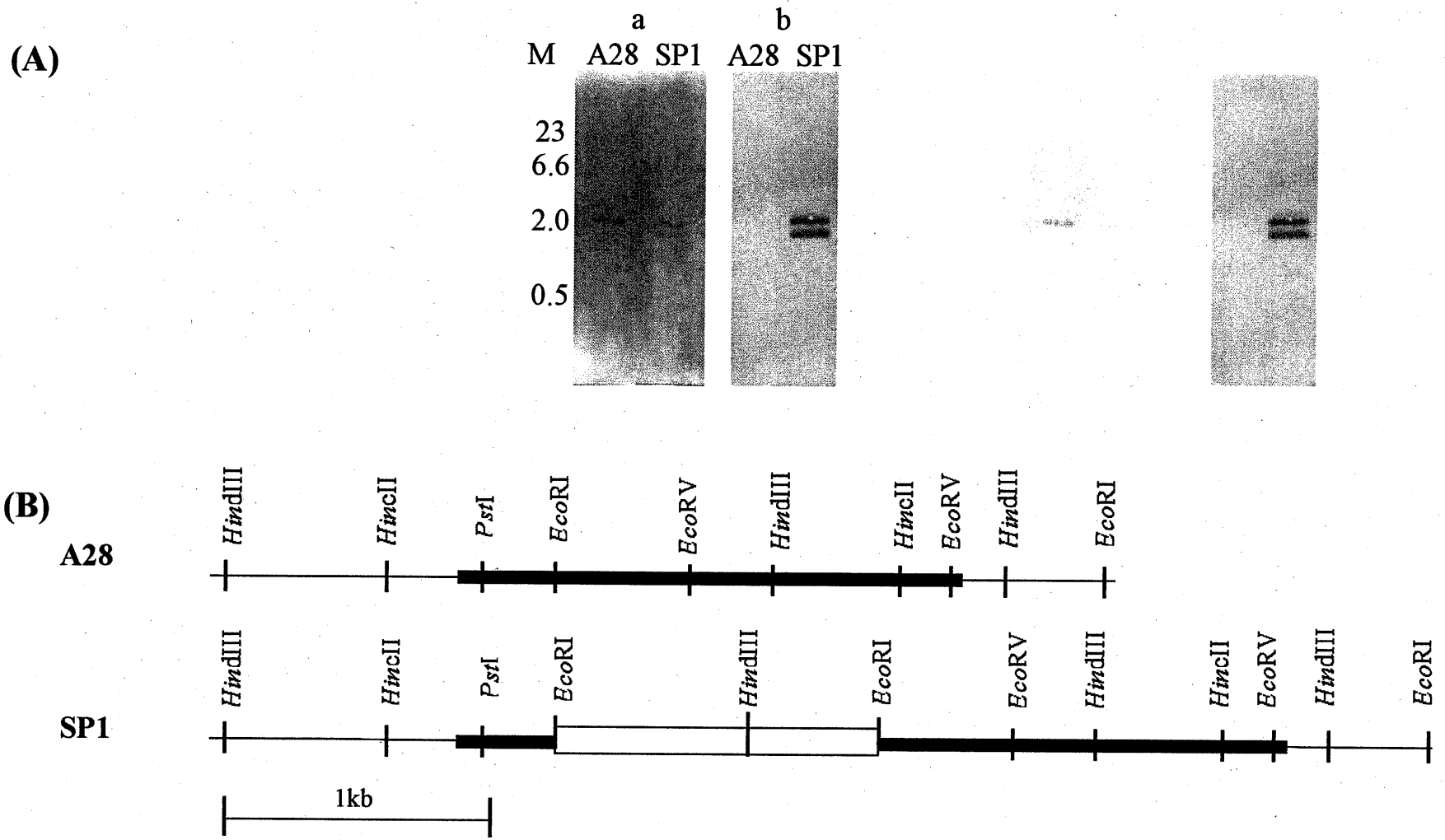

Fig. 4.6. Comparison of *aspI* gene DNA regions of strain A28 and AspI-inactivated mutant SP1. (A) Southern blot of *Hin*dIII digested chromosomal DNA using probe of *Hin*dIII 2.1-kb fragmets (a) and Km^r cassette (b). (B) Restriction maps of the *aspI* gene regions of strain A28 and mutant strain SP1.

culture supernatants added, respectively. The algicidal effect of SP1 culture supernatant was pretty reduced but not diminished completely.

4.4 Conclusion and discussions

Molecular cloning and sequencing of the serine protease gene, *aspI*, revealed an open reading frame of 2,073 bp with the capacity to encode a polypeptide of 691 amino acids and with a molecular size of 71,007. The molecular mass of mature AspI is 50 kDa by SDS-PAGE electrophoresis analysis, suggesting that the protease is synthesized as a pre-protein composed of four domains: the signal sequence, the N-terminal pro-region, the mature AspI, and C-terminal extension. The C-terminal regions were characterized by two repeated sequences, which showed high sequence similarities with those of the C-terminal pro-regions from other known bacteria, such as *Alteromonas* sp. (68), *Xanthomonas campestris* (37), *Vibrio cholerae* (22), *Vibrio vulnificus* (43) with 73 %, 61 %, 60.8 %, and 57% amino acid identity, respectively (Fig. 4. 5). These proteases are produced the mature protein by processing the C-terminal pro-regions, but strain A28 AspI is thought to be excreted having one of the repeated sequence of C-terminal region. The functional domains of *V. vulnificus* metalloprotease were reported, and the C-terminal polypeptide is suggested to be essential for efficient attachment to insoluble protein substrates (43). It has been reported that *Rarobacter faecitabidus* produced an extracellular protease with yeast-lytic activity (57). The *R. faecitabidus* protease was a chimera of a serine protease on the NH2 –terminal side and a mannose-binding domain with a lectin-like affinity for mannose on the COOH-terminal side. When the mannose-binding domain was truncated, the mutant protease showed normal protease activity but failed to lyse yeast cells. The C-terminal region peptides of AspI may be mediate efficiency of association with the target substrate, *S. costatum* cells.

The *aspI* gene inactivated mutant strain, SP1, was isolated by insertion of kanamycin resistance gene. SP1 showed reduction of extracellular protease activity compared with the parental strain A28 but still produced extracellular protease(s). When PMSF, serine protease inhibitor, and *1,10*-phenanthroline, metalloprotease inhibitor, were added to culture supernatant of strain A28, the protease activities were diminished 85% and 20% respectively. Strain A28 produced at least two kinds of extracellular proteases including the serine and metal proteases. Strain SP1 culture supernatant protease activity may be due to another serine protease, AspII and metalloprotease. The algicidal activity of SP1 culture supernatant was dramatically decreased. This result makes clear that extracellular serine protease AspI of A28 is the major algicidal substance and strain A28 is thought to produce substance which responsible for the algicidal effect except for AspI.

Chapter 5.

Cloning and Characterization of Algicidal Metalloprotease Gene of *Pseudoalteromonas* sp. Strain A28.

5.1 Introduction

The culture supernatant of *Pseudolateromonas* sp. strain A28 had algicidal activity. Strain A28 produces an extracellular serine protease, which was responsible for the algicidal activity of this marine bacterium. The purified algicidal protease, AspI, was a serine protease and a monomeric protein with a molecular mass of 50 kDa. Addition of PMSF, which is serine protease inhibitor, diminished algicidal activity of the A28 culture supernatant, however, it did not result in a complete loss of algicidal activity. AspI inactivated mutant strain, SP1, was isolated and detected the weak algicidal activity. These results suggest that existence of algicidal substance(s) but AspI in A28 culture supernatant. Another serine protease was also purified, named AspII, which was not detected the algicidal effect. Strain A28 secretes not only serine proteases but also metalloprotease. When, *1,10*-phenanthroline, which is metalloprotease inhibitor, was added to culture supernatant of strain A28, the protease activity was decreased 20 % of total activity. This result prompted to search another extracellular protease, which has algicidal activity other than A28 AspI. This chapter is presented molecular cloning of the metalloprotease encoding gene from *Pseudolateromonas* sp. strain A28 chromosomal DNA. Cloned metalloprotease gene was sequenced, expressed in *E. coli.*, and

purified. Purified metalloprotease is characterized and investigated the algicidal effects on *S. costatum* cells.

5.2 Materials and methods

5.2.1 Bacterial strains and plasmids

E. coli HB101 (1) was used for construction of the genomic library of *Pseudoalteromonas* sp. strain A28. *E. coli* strains were grown at 37°C with shaking in LB medium. Whenever necessary, ampicillin and kanamycin were added at final concentrations of 100 and 200 $\mu g ml^{-1}$, respectively.

5.2.2 Construction of genomic library

Strain A28 genomic library was constructed by ligating 4- to 7-kb *Sau*3AI partially digested DNA fragments to the *Bam*HI site of calf intestine alkaline phosphatase (Takara shuzo, Tokyo) dephosphorylated pUC118 (70). The ligated DNA fragments were used to transform host strain *E. coli* HB101. Transformants were screened for protease activity by formation of clear zones around the colonies on the LB agar plate containing 1% Bacto skim milk (Difco) and 100 $\mu g\ ml^{-1}$ Amp.

5.2.3 DNA manipulation and sequencing

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligation, transformations, agarose gel electrophoresis, and Southern hybridization (51). The nucleotide sequence of both strands was determined by the dideoxynucleotide sequencing method with single-stranded DNA (52). Nucleotide and deduced amino acid sequences were analyzed and compared with other known sequences in the GenBank library of DNA sequences by use of

DNASIS programs (Hitachi Software Engineering, Kanagawa, Japan) and the FASTA program (48).

5.2.4 Enzyme purification

HB101 [pPEMP01.1] cultures were grown for 4 days at 30°C in LB medium containing 200 μg ml^{-1} of ampicillin, and the cells were harvested by centrifugation at 6,500 × *g* for 15 min. The culture supernatant was filtered through a 0.45-μm nitrocellulose membrane filter (Advantec Toyo, Tokyo) to remove any remaining bacteria. The proteins were precipitated by adding solid ammonium sulfate to 80 % saturation in the ice bath. The precipitate was redissolved in TM buffer (20mM Tris-HCl (pH7.8), 2mM $MgCl_2$) and dialyzed against TM buffer. The dialysate was applied to an anion-exchange column (Poros HQ/M, 4.6 x 100mm; PerSeptive Biosystems Inc., Framingham, Mass.). The column was washed with TM buffer, and proteins were eluted with a linear NaCl gradient of 0 to 1 M in TM buffer. Fractions with high metal protease activities were collected and dialyzed against 50mM phosphate buffer (pH7.0) containing 1 M ammonium sulfate. The dialyzed sample was then loaded to a hydrophobic interaction column (Poros PH, 4.6 x 100 mm; PerSeptive Biosysytems Inc.) equilibrated with 50mM phosphate buffer (pH 7.0) containing 1M ammonium sulfate. The column was developed with a linear gradient of 1 to 0 M ammonium sulfate. Active fractions were pooled and concentrated by ultrafiltration. Then, preparative native electrophoresis was performed using a Mini Prep Cell (Bio-Rad) as described previously (13). Purity of the protease was determined by electrophoresis on SDS polyacrylamide electrophoresis (SDS-PAGE) gels. Protein bands were visualized by using the silver stain method. The N-terminal amino acid sequence was determined with a Precise protein sequencing system (Applied Biosystems, Foster City, Calf)

5.2.5 Enzyme assays

To assess protease activity, a reaction mixture (0.5 ml) containing 10mg of Azocasein (Sigma) and enzyme solution appropriately diluted in TM buffer (20 mM Tris-HCl (pH7.8), 2 mM $MgCl_2$), was incubated at 30℃for 30 min. One unit of protease activity was defined as the amount of enzyme that caused an increment of one absorbance unit per hour. In addition, protease activity of separated proteins in a SDS-polyacrylamide gel was detected by co-polymerizing 0.5% skim milk in the polyacrylamide matrix. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 30min at 4 °C, incubated at 37 °C for 1h in TM buffer, and then fixed and stained with 0.5% Brilliant Blue R in ethanol-acetic acid-water (2:1:7, v/v/v). After the gel was destained with the same solution without the dye, protease activity was detected by measuring zones of clearing in the SDS-skim milk-polyacrylamide gel. The algicidal activity of protease was determined by a paper disk assay. Algicidal activity was assessed by the presence of clear zones around the paper disks.

Effect of protease inhibitors were measured by using phenylmethylsulfonyl fluoride (PMSF; Sigma), diisopropylphosphoro fluoridate (DFP; Katayama, Osaka), *1,10*-phenanthroline (Sigma), EDTA (Sigma), tetraethylenepentamine, and ethylene glycol bis (β -aminoethylesther)-*N,N,N',N'*-tetraacetic acid (EGTA; Sigma). The mixture of each protease inhibitor and enzyme solution appropriately diluted in 250mM Tris-HCl (pH7.8) was incubated at 20 °C for 30 min before Azocasein was added.

The pH effect of protease activity was determined by using 50 mM acetate buffer at pHs of 4.6 and 5.0, 50 mM phosphate buffer at pHs of 6.0 and 7.0, and 50 mM Tris buffer at pHs of 7.8, 8.6, 9.4 and 10.6. Protease activity was measured at 30°C. For, temperature effect testing, the purified protein activity was measured various temperatures at 4, 10, 20, 30, 40, 50, 60, 80°C. Protease activity was assessed by the method described above.

5.3 Results

5.3.1 Cloning of the *Pseudoalteromonas* sp. strain A28 metalloprotease gene

Strain A28 genomic library was constructed in *E. coli* HB101 using the plasmid vector pUC118. Transformants were screened for protease activity by selection on LB agar plates containing 1% Bacto skim milk (Difco) and 100 μg ml^{-1} Amp. From a screen of approx. 30,000 colonies, three clones were selected which exhibited zones of clearing. Plasmid DNA was isolated from each of the three clones and characterized by restriction enzyme analysis. All of the recombinant plasmids were shown to contain overlapping DNA insert fragments. A single plasmid (pPEMP01) was selected for further analysis. The genomic origin of the pPEM01 insert was confirmed by Southern blot analysis of A28 chromosomal DNA. A map of pPEMP01 was constructed and showed that the plasmid contained 5.2-kb of A28 chromosomal DNA. The 5.2-kb insert of pPEMP01 was digested with various restriction enzymes, and the fragments were subcloned into pUC118 to make pPEMP01.1, pPEMP01.2, and pPEMP01.3 (Fig. 5.1). When *E. coli* HB101 cells were transformed with pPEMP01.1, the transformant formed a clear zone on LB agar plate supplemented with Bacto milk and ampicillin. Neither *E. coli* HB101 [pPEMP01.2] nor *E. coli* HB101 [pPEMP01.3] showed proteolytic activity. Thus, the gene encoding the protease was localized to a 2.8-kb *Sau*3AI-*Pst*I fragment of pPEMP01. The protease-encoding gene, encoded for a putative protein of 731 amino acids with a molecular weight of 77,784. The N-terminal amino acid sequence of the purified A28 AspI, previously determined by protein sequencing, was not find in the deduced amino acid sequence protease gene was localized within a 2.8-kb *Sau*3AI-*Pst*I fragment of pPEMP01.

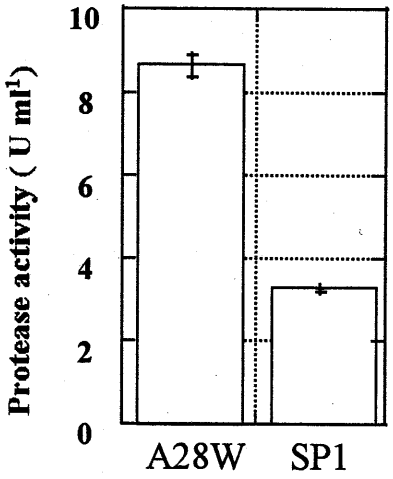

Fig. 4.7. Comparison of culture supernatant strain A28 and the AspI-inactivated mutant SP1. Protease activity of overnight culture supernatants were measured as azocasein hydrolytic activity.

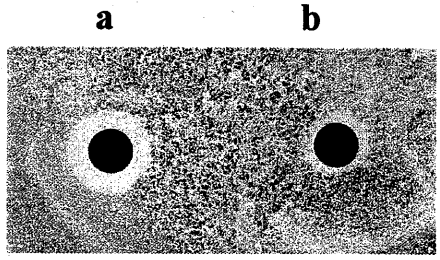

Fig. 4.8. Detection of culture supernatant algicidal activity of strain A28 and AspI-inactivated strain SP1. The overnight cultures were added on the *S. costatum* lawn. a, strain A28 (20 _l); b, SPI (20 _l)

5.3.2 DNA sequence analysis

Nucleotide sequence analysis of the pPEMP01.1 insert revealed that the 2.8-kb *Sau*3AI-*Pst*I fragment contained an open reading frame (ORF), which was designated of the *empI* gene. A computer-assisted sequence similarity search revealed substantial similarity (51.8% identity in a 498-amino-acid overlap) between EmpI and the *Pseudomonas aeruginosa* elastase precursor (16). Elastase is a zinc metalloprotease mediates tissue destruction during *Pseudomonas* infection (23). *P. aeruginosa* elastase is initially translated as a 498 amino acid polypeptide and processed during export to a mature polypeptide of 301 amino acids. A 304 amino acid EmpI protein commencing with Ala-207 showed 61.2% identity with the mature *P. aeruginosa* elastase. The putative protein also had 62.5, 63.0, and 64.4% amino acid identity with the *Vibrio cholerae* HA/protease (8) the *V. vulnificus* metal protease (7), and the *V. proteolyticus* vibriolysin (9), respectively. The crystal structure of the *P. aeruginosa* elastase has been established (67) and the zinc-binding sites, the calcium-binding sites and the active sites have been determined. All of the sites were conserved in the EmpI protein (Fig. 5.2A). The deduced C-terminal amino acid sequence of EmpI exhibits significant similarity to the C-terminal of alkaline serine protease I of the marine bacterium *Alteromonas* sp. strain O-7 which undergoes C-terminal processing (68). The C-terminal 219 amino acids of alkaline serine protease I and EmpI share 48.4 % amino acid identity (Fig. 5.2B).

5.3.3 Purification and characterization of metalloprotease

To further characterize the protease encoded by the *empI* gene, the protease was purified to homogeneity from the culture supernatant of HB101 [pPEMP01.1] by ion exchange and hydrophobic interaction chromatography, followed by preparative gel electrophoresis (Table 5.1). The N-terminal amino acid sequence of the purified

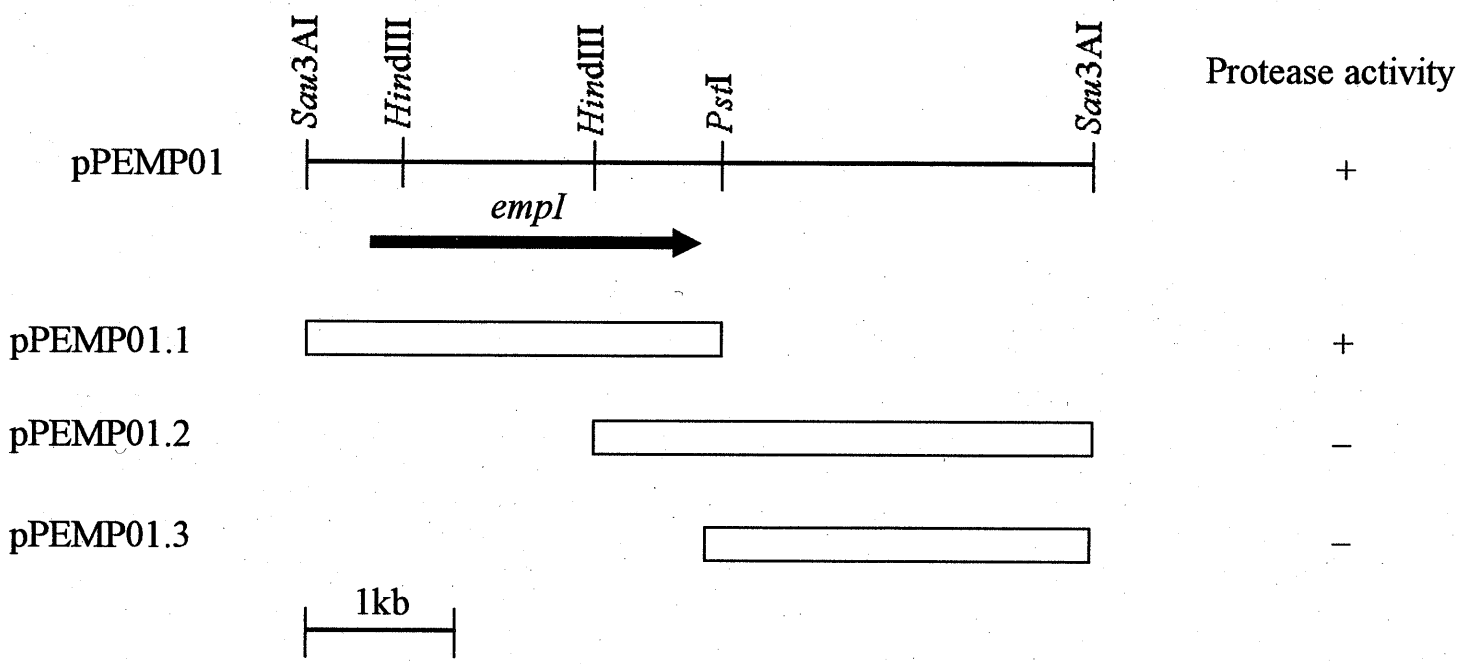

Fig. 5.1. Restriction map of plasmid pPEMP01 containing the 5.2-kb *Sau*3AI fragment of the strain A28 chromosomal DNA and its subclones. The light part summerizes whether the plasmid enabled HB101 to form clear zones around its colonies on LB plate containing skim milk.

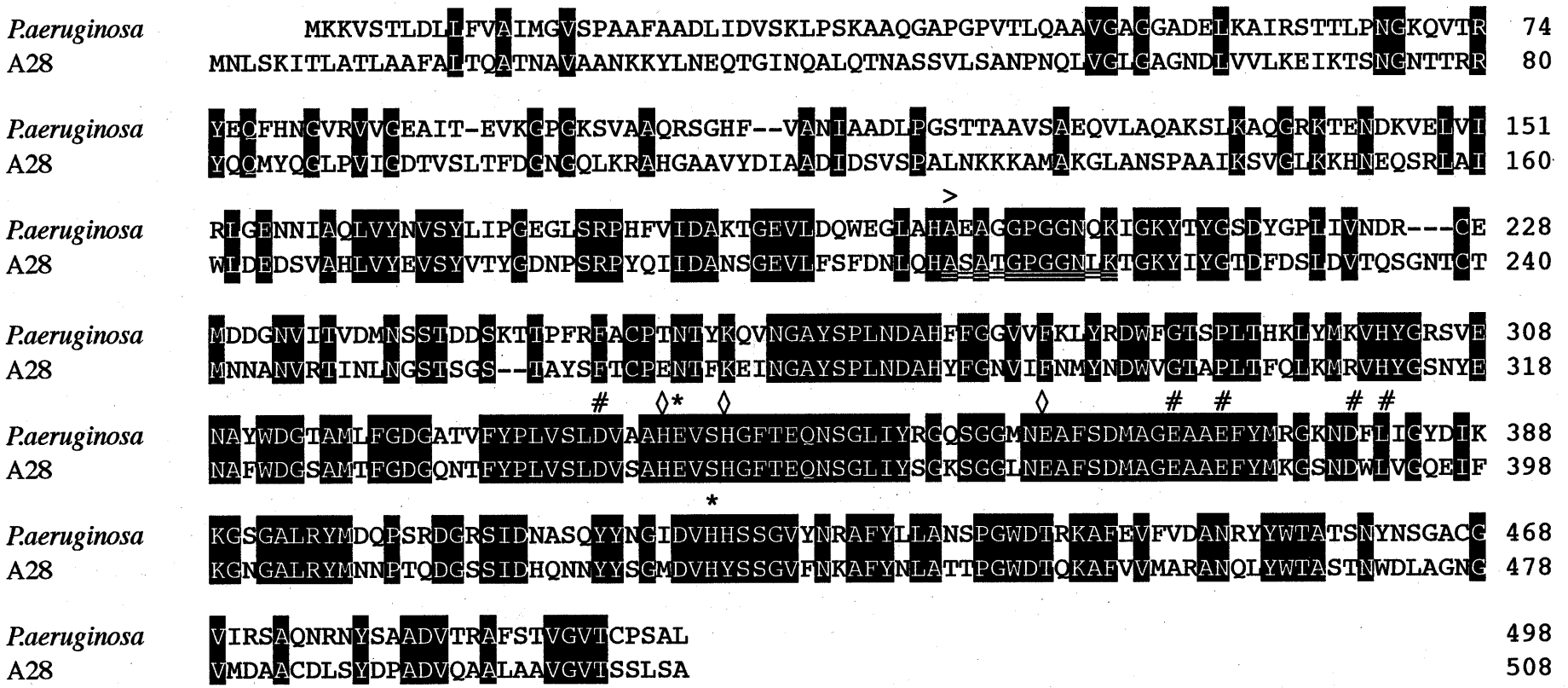

Fig. 5.2. Amino acid sequence of EmpI deduced from open reading frame. (B). Comparison of deduced C-terminal amino acid sequence of EmpI with *Alteromonas* sp. strain O-7 extracelluar protease AprI C-terminal pro-region

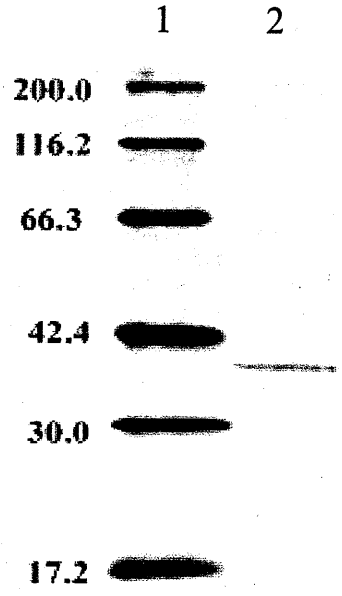

Fig. 5.3. SDS-PAGE analysis of purified EmpI from *E.coli*. Protein pooled from the purification step of preparative native-protein gel electrophoresis was subjected to electrophoresis and silver stained. Lane 1, molecular mass markers; lane 2, purified EmpI (2 μg)

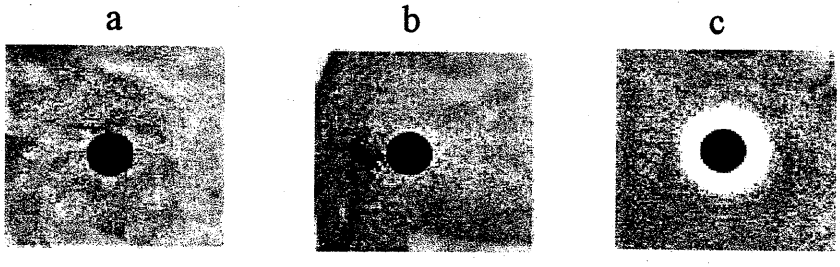

Fig. 5.4. Detection of Algicidal activity of EmpI. Zones of clearing around paper disks indicate the lysis of the diatom *S. costatum* incubated for 24hr. a : preparative native-protein gel electrophoresis buffer (20 μl), b: EmpI rich fraction from preparative native-protein gel electrophoresis (0.16 U of protease activity), c : EmpI rich fraction from preparative native-protein gel electrophoresis (0.32 U of protease activity).

protein was Ala-Ser-Ala-Thr-Gly-Pro-Gly-Gly-Asn-Leu-Lys, which corresponds to the amino acid sequence from position 207 to 217 (Fig. 5.2A). By means of SDS-PAGE, the molecular mass of the EmpI protease was estimated as 38kDa. (Fig. 5. 3) Since the molecular weight of the EmpI protease was also estimated as 38kDa by gel filtration, the EmpI protease should be monomer. Paper disk assays confirmed that the purified protease, EmpI, had algicidal effects on *S. costatum* (Fig. 5.4).

Inhibition experiments of the purified protease were performed with possible inhibitors. *1,10*-phenanthroline, which is inhibitor of metalloprotease, severely inhibited the activity of the protease (Table 5.2). No significant inhibition was detected with the serine protease inhibitors including PMSF and DFP. These results suggest that the purified protease is a metalloprotease.

To investigate whether strain A28 produces EmpI protease or not, extracelluar supernatant of A28 was analyzed using SDS-skim milk-PAGE gels (Fig. 5.5). Five major bands (117, 75, 59, 50, and 47 kDa) and three minor bands (130, 64, 38 kDa) of protease activity were present in the supernatant fraction. When extracelluar supernatant was treated with 1mM PMSF before electrophoresis, the major bands of protease activity disappeared. In the previous chapter described the presence of two serine protease, AspI, and AspII in extracelluar supernatant of A28. AspI had a molecular mass of 50 kDa and AspII, which did not show any algicidal activity, had a molecular mass of 75 kDa. Therefore, the 50 kDa and 75 kDa bands, which were sensitive to PMSF, were supposed to correspond to AspI and AspII. The 38 kDa band co-migrated with the purified EmpI protease. These results suggest that the 38 kDa band in extracelluar supernatant corresponds to the EmpI protease. The 38 kDa band was resistant to PMSF, while this band was not detected in extracelluar supernatant after treated with *1,10*-phenanthroline. These results suggest that the 38 kDa band in extracelluar supernatant of A28 corresponds to EmpI.

Table 5. 1. Purification of Metalloprotease from the Culture Supernatant of *E. coli* HB101 [pPEMP01.1]

Sample analyzed	Total protein	Total activity	Specific activity	Purification	Yield
	mg	*units*	*units/mg*	*fold*	*%*
Supernatant	60.84	448	7	1	100
Salted-out	20.75	155	7.5	1.07	34
Anion-exchange column	4.10	103	25	3.57	23
Hydrophobic column	0.39	19	48	6.85	4
Preparative electrophoresis	0.11	7.2	66	9.4	1.6

Table 5.2. Inhibitor effects on the protease activity of the purified EmpI

Compound (conc)	Relative activity (%)
None	100
EDTA (1mM)	52
EGTA (1mM)	33
1,10-phenantroline (1mM)	3
tetraethylenepentamine (1mM)	78
PMSF (1mM)	110
DFP (1mM)	115

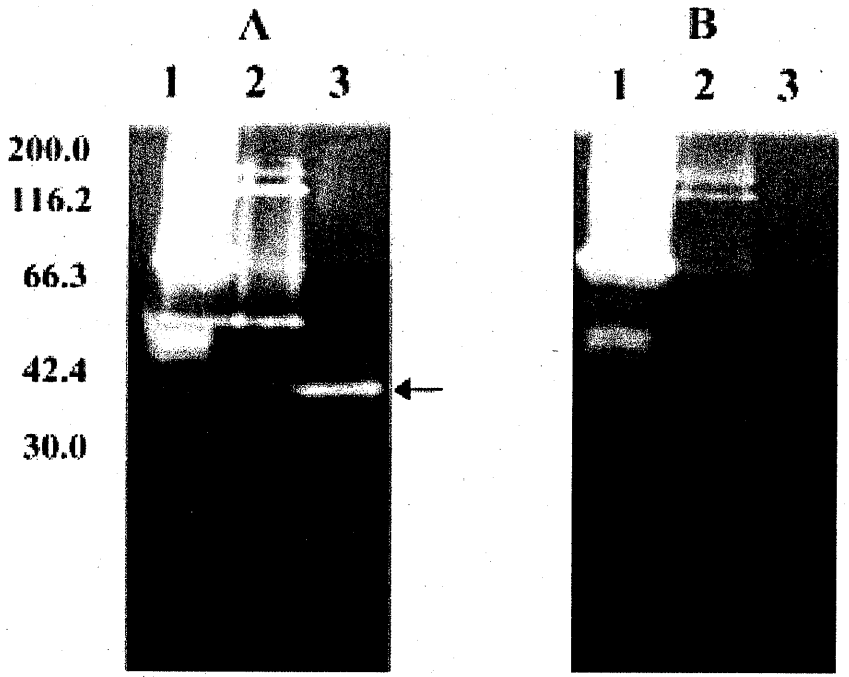

Fig. 5.5. Proteolytic Analysis of the Extracelluar Supernatant Proteins from Strain A28 and Purified EmpI. Protease with SDS-Skim Milk-PAGE. SDS-skim milk-PAGE gels were stained after removal of SDS by soaking in Triton X-100 then incubated in 50mM Tris-HCl buffer (pH7.8) (A) and in 50mM Tris-HCl buffer (pH7.8) with 5mM 1,10-phenanthroline. (B) respectively. Lane 1, extracelluar supernatant of A28; lane 2, extracelluar supernatant of A28 treated with 1mM PMSF before electrophoresis; lane 3, purified EmpI protease. Arrow indicates purified EmpI protease activity.

5.4 Conclusion and discussions

Pseudoalteormonas sp. strain A28 showed culture supernatant proteases activities produced two kinds of extracelluar proteases, serine and metal proteases by the investigation of protease inhibitors. The protease activity of the A28 culture supernatant was inhibited by PMSF and *1,10*-phenanthroline, about 85% and 20% of protease activity were vanished respectively. When both inhibitors PMSF and *1,10*-phenanthroline were added A28 culture supernatant at the same time, protease activity disappeared. Previously purified algicidal product from A28 culture supernatant, which is a serine protease designated AspI. In this chapter demonstrated cloning of the metalloprotease gene *empI* then expressed in *E. coli* and purified. AspI, which was shown to be responsible for the algicidal activity of this marine bacterium, is a serine protease. The N-terminal amino acid sequence of AspI was not found in the deduced amino acid sequence of the *empI* gene. Therefore, EmpI is an algicidal protease distinct from AspI.

Both purified proteases AspI and EmpI degraded azocasein, but no elastolytic activity was detected with elastin Congo red substrate. The C-terminal pro-region amino acid sequence of EmpI was showed 44% identity with AspI C-terminal region. Whereas AspI was produced into extracellular fraction with that C-terminal region, C-terminal region of EmpI was thought to be processed by the molecular weight of 38 kDa at SDS-PAGE analysis and zymogram analysis. The purified metalloprotease, EmpI showed algicidal activity when supplied paper disk on the *S. costatum* lawn but EmpI needs twofold protease unit, which is correspond to 6.2 fold protein concentration of AspI to show algicidal activity. This may suggest that EmpI had substrate specificities different from those of AspI. Alternatively, they may have different affinities for *S. costatum* cells. The affinity of the protease for algal cells should be of importance for causing the algicidal effects.

Chapter 6.

Conclusions and Future Scope

In this study, algicidal mechanism of algicidal marine bacterium was investigated by genetic and biochemical approaches. The marine bacterium *Pseudoalteromonas* sp. strain A28 is able to kill the diatom *Skeletonema costatum* NIES-324. The important results of this study as follows. These results may be useful to understand the algicidal mechanism of bacteria, which would assist to understand the interactions of algal blooms and the bacteria.

Strain A28 produced extracellular algicidal substance, which is heat sensitive and a molecular mass of over than 10,000-M_w. The culture supernatant of A28 was found to have protease and DNase activities. The algicidal marine bacteria can generally be divided into two groups by their algicidal mechanisms. The first group, directly attacks and lyses target algal cells after cell-to-cell attachment, and the second group produces and excretes algicidal substances to kill algal cells. Strain A28 did not need to cell-to-cell contact to kill algal cells.

Algicidal substance of strain A28 was purified. The extracellular serine protease, AspI had a molecular weight of 50 kDa. Another extracellular serine protease of A28, AspII was also purified but algicidal activity was not detected. Commercially avabile proteases such as trypsin, pepsin, subtilisin, and pronase did not showed algicidal effects. The algicidal substance, AspI was thought to have not only protease activity but also have any function need to exhibit algicidal activity.

AspI was a major algicidal product of strain A28 culture supernatant. Molecular cloning and sequencing of *aspI* gene was performed. Then, AspI-inactivated mutant strain, SP1 was constructed by the Km^r gene integration into the AspI sequences on the chromosomal DNA of strain A28. The culture supernatant of SP1 showed dramatically reduced algicidal activity. This result suggested that AspI is a

main algicidal substance in the culture supernatnat of A28.

Strain A28 produced extracelluar serine proteases and also, metalloprotease into culture fractions. Addition of PMSF which is serine protease inhibitor, diminished algicidal activity of the A28 culture supernatant, however, it did not result in a complete loss of algicidal activity. The protease activity of strain A28 culture supernatant was inhibited by PMSF and *1,10*-phenantroline which is metalloprotease inhibitor, about 85% and 20% of protease activity were vanished respectively.

Metalloprotease gene of strain A28 was cloned, sequenced, and expressed in *E. coli*. Purified metalloprotease, EmpI showed algicidal acitivity. EmpI needs twofold protease unit, which is correspond to 6.2 fold protein concentration of AspI to show algicidal activity. This may suggest that EmpI had substrate specificity different from those of AspI. Alternatively, they may have different affinities for *S. costatum* cells. The affinity of the protease for algal cells should be important for causing the algicidal effects. EmpI was a minor protease in the extracellular proteases of strain A28.

In this study, algicidal effects of algicidal marine bacterium *Pseudoalteromonas* sp. strain A28 was investigated. The culture supernatnat of strain A28 had algicidal activity. The major algicidal substance of srtain A28 was purifed and identifed. The purified algicidal substance extracellular serine protease. The serine protease is thought to have affinity for algal cells and/or substrate specificity to exhibit algicidal activity. Affinity and substrate specificity of algicidal substance to algal cells may be important point to understaning of algicidal activity. And analysis of fuctional structure of algicidal substance will be needed to clarify the algicidal mechanism.

As shown results of this study, the identified and purified algicidal substances from strain A28 will be further useful for understanding relationships of bacteria and algal cells in marin ecosystem.

References

1. Amemura, M., Makino, K., Shinagawa, H., Kobayashi, A., and Nakata, A. 1985. Nucleotide sequence of the genes involved in phosphate transport and regulation of the phosphate regulon in *Escherichia coli*. J. Mol. Biol. 184:241-250.
2. Aoyagi, T., Takeuchi, T., Matsuzaki, A., Kawamura, K., Kondo, S., Hamada, M., Maeda, K., and Umezawa, H. 1969. Leupeptins, new protease inhibitors from *Actinomycetes*. J. Antibiot. (Tokyo) 22:283-286.
3. Baker, K. H., and Herson, D. S. 1978. Interactions between the diatom *Thallasiosira pseudonanna* [sic] and an associated pseudomonad in a mari-culture system. Appl. Environ. Microbiol. 35:791-796.
4. Blaschek, H. P., and Klacik, M. A. 1984. Role of DNase in recovery of plasmid DNA from *Clostridium perfringens*. Appl. Environ. Microbiol. 48: 178-181.
5. Burnharm, J. C., Collart, S. T., and dfat, M. J. 1984. Myxococcal predation of the cyanobacterium *Phormidium luridum* in aqueous environments. Archieves of Microbiology 137: 220-225.
6. Chen, L. C. M., Edelstein, T., and MacLachlin, J. 1969. *Bonnemaisonia hamifera* Hariot in nature and in culture. J. Phycol. 5:211-220.
7. Cheng, J. C., Shao, C. P., and Hor, L. I., 1996. Cloning and nucleotide sequencing of the protease gene of *Vibrio vulnificus*. Gene 183:255-257.
8. Claudia C. Hase, and Richard A. Finkelstein. 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemaggultinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J. bacteriol. 173:3311-3317.
9. David, V. A., Deutch, A. H., Sloma, A., Pawlyk, D., Ally, A., and Durham, D. R. 1992. Cloning, sequencing and expression of the gene encoding the extracellular neutral protease, vibriolysin, of *Vibrio proteolyticus*. Gene 112:107-112.
10. Dixon, G. H., Kaufman, D. L., and Neurath, H. 1958. Amino acid sequence in the region of diisopropylphosphoryl binding in diisopropylphosphoryl-trypsin. J.

Biol. Chem. 233:1373-1381.

11. Doucette, J. G. 1995. Interactions between bacteria and harmful algae: a review. Nat. Toxins. 3: 65-74.

12. Egan, S., James, S., Holmtrom, C., and Kjelleberg, S. 2001. Inhibition of algal spore germination by the marine bacterium *Pseudoalteromonas tunicata*. FEMS Microl. Ecol. 35:67-73.

13. Feldman, D. A., and Weinhold, P. A. 1998. Cytidylytransferase-binding Protein is identical to transcytosis-associated protein (TAP/p115) and enhances the lipid activation of cytidylyltransferase. J. Biol. Chem. 273:102-109.

14. Fukami, K., Yuzawa, A., Nishijima, T., and Hata, Y. 1992. Isolation and properties of a bacterium inhibiting the growth of *Gymnodinium nagasakiense*. Nippon Suisan Gakkaishi 58:1073-1077.

15. Fukami. K., Sakaguchi, K., Kanou, M., Nishijima, T. 1996. Effect of bacterial assemblages on the successtion of blooming phytoplankton from *Skeletonema costatum* to Heterosigma akashiwo. In: Yasumoto, T., Oshima, T., Fukuyo, Y. (eds.) Harmful and toxic algal blooms, Intergov. Oceanogr. Comm. Of UNESCO, pp. 335-338.

16. Fukushima, J., Yamamoto, S., Morihara, K., Atsumi, Y., Takeuchi, H., Kawamoto, S., and Okuda, K. 1989. Structural gene and complete amino acid sequence of *Pseudomonas aeruginosa* IFO 3455 elastase. J. Bacteriol. 171:1698-1704.

17. Furuki, M., and Kobayashi, M. 1991. Interaction between *Chattonella* and bacteria and prevention of this red tide-EMECS '90. Mar. Pollut. Bull. 23: 189-193.

18. Gauthier, G., Gauthier, M., and Christen, R. 1995. Phylogenetic analysis of the genera *Alteromonas*, *Shewanella* and *Moritella* using genes coding for small-subunit r RNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal

of twelve new species combinations. Int. J. Syst. Bacteriol. 45:755-761.

19. Gold, A., and Fahrney, D. 1964. Sulfonyl fluorides as inhibitors of esterase. II. Formation and reactions of phenylmethylsulfonyl a-chymotrypsin. Biochemistry 3:783-791.

20. Hallegraeff, G. M. 1993. A review of Harmful algal blooms and their apparent global increase. Phycologia 32:79-99.

21. Hanahan, D. 1983. Studies on Transformation of *Escherichia coli* with Plasmids. J. Mol. Biol. 166:557-580.

22. Hase, C. C., and Finkelstein, R. A. 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J. Bacteriol. 173:3311-3317.

23. Holder, I. A., and Neely, A. N. 1989. Pseudomonas elastase acts as a virulence factor in burned hosts by Hageman factor-dependent activation of the host kinin cascade. Infect. Immun. 57:3345-3348.

24. Imai et al. 1993. Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal sea of Japan. Mar. Biol. 116:527-532.

25. Imai, I., Kim, M. C., Nagasaki, K., Itakura, S., Ishida, Y. 1998. Detection and euneration of killer microorganisms against harmful phytoplankton in the costal sea. Plankton Biol. Ecol. 45:15-25.

26. Imai, I., Ishida, Y., Sawayama, S., and Hata, Y. 1991. Isolation of a marine gliding bacterium that kills *Chatonella antiqua* (Raphidophyceae). Nippon Seuisan Gakkaishi 57:1409.

27. Imai, I., Ishida, Y., Sakaguchi, K., and Hata, Y. 1995. Algicidal marine bacteria isolated from northern Hiroshima bay, Japan. Fisheries Science 61: 618-636.

28. Ishio, S., Mangindaan, R.E., Kuwahara, M., Nakagawa, H. 1989. A bacterial hostile to flagellates: identification of species and characters. In: Okaichi, T., Anderson, D. M., Nemoto, T. (eds) Red tides: Biology, environmental Science, and Toxicology, Elsevier Sci. Pub. Co., Inc., N.Y., pp. 205-208.

29. Kato, J., Amie, J., Murata, Y., Kuroda, A., Mitsutani, A., and Ohtake, H. 1998. Development of a genetic transformation system for an Alga-lysing bacterium. Appl. Environ. Microbiol. 64: 2061-2061.

30. Katser, H., 1979. Growth interaction between marine dinoflagellates in multispecies culture experiments. Mar. Biol. 52:357-369.

31. Kim, M. C., Yoshinaga, I., Imai, I., Nagasaki, K., Itakura, S., Ishida, Y. 1998. A close relationship between algicidal bacteria and termination of *Heterosigma akashiwo* (Rsphidophyceae) blooms in Hiroshima Bay, Japan. Mar. Ecol. Prog. Ser. 170:25-32.

32. Kozak, M. 1983. Comparison of Initiation of Proteins Synthesis in Procaryotes, Eucaryotes, and Organelles. Microbiol. Rev. 47:1-45.

33. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

34. LaPointe, B. E., and O'conell, J. D. 1989. Nutrient-enhanced growth of *Cladophora prolifera* in Harrington Sound, Bermuda: eutrophication of a confined, phosphorus-limited marine ecosystem. Estur. Coast. Shelf Sci. 28:347-360.

35. Lee,M.A., Liu,Y.C. 2000. Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei*. FEMS Microbiol. Lett. 192:67-72.

36. Lilley, G. G, Riffkin, M. C., Stewart, D. J., Kortt, A. A., 1995. Nucleotide and deduced protein sequence of the extracellular, serine basic protease gene (*bprB*) from *Dichelobacter nodosus* strain 305: comparison with the basic protease gene (*bprV*) from virulent strain 198. Biochem. Mol. Biol. Int. 36:101-112.

37. Liu, Y. N., Tang, J. L., Clark, B. R., Dow, J. M., and Danniels, M. J. 1990. A smultipurpose broad host range cloning vector and its use to characterization an extracellular protease gene of *Xanthomonas campestris* pathovar *campestris*. Mol.Gen. Genet. 220:433-440.

38. Lovejoy, C., Bowman, J. P., and Hallegraeff, G. M. 1998. Algcidal effects of a novel marine *Pseudoalteomonas* isolate (class Proteobacteria, gamma subdivision) on harmful algal bloom species of the genera *Chattonella, Gymnodinium*, and *Heterosigma*. Appl. Environ. Microbiol. 64:2806-2813.

39. Maeda, T., Murakami, M., Ohsugi, S., Furushita, M., Mitsutani, A., and Shiba, T. 1998. Perspectives of the development of 16S rDNA probe specific for algicidal and/or algal-lytic gliding bacteria. Fisheries Science 64: 861-865.

40. Maniatis, T. , Fitsch, E. F., and Sambrook, J. 1982. Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Habor, N. Y.

41. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.

42. Mitsutani, A., Yamasaki, I., Kitaguchi, H., Kato, J., Ueno, S., and Ishidaa, Y. 2001. Analysis of algicidal proteins of a diatom-lytic marine bacterium *Pseudoalteromonas* sp. strain A25 by two-dimentional electrophoresis. phycologia 200 40:286-291.

43. Miyoshi, S., Wakae, H., Tomochika, K., and Shinoda, S. 1997. Functional domains of zinc metalloprotease from *Vibrio vulnificus*. J. Bacteriol. 179:7606-7609.

44. Mizsutani, A., Takesue, K., Kirita, M., and Ishida, Y. 1992. Lysis of *Skeletonema costatum* by *Cytophaga* sp. isolation from the costal water of the Ariake Sea. Nippon Seuisan Gakkaishi 58: 2159-2167.

45. Ohsawa, K., and Ebata, N. 1983. Silver stain for detecting 10-femtogram quantities of protein after polyacrylamide gel electrophoresis. Anal. Biochem. 135:409-415.

46. Okaichi, T. 1987. Science of Algal blooms. In Fukuyo, Y. and Takahashi, S., Biological Characterization of red tide algae. Kakihoshi publishing Co. Tokyo, pp. 37-89.

47. Okaichi, T. 1997. Red tide in the Seto Inland sea. In okaichi, T. and Yanagi, T.

(eds), sustainable development in the Seto Inland sea, Japan-from the viewpoint of Fisheries. Terra Scientific publishing Co., Tokyo, pp. 251-304.

48. Pearson, W. R., and Lipman, D. J. 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA 85:2444-2448.

49. Romalde, J. L., Toranzo, A. E., and Barja, J. L. 1990. Changes in bacterial populations during red tides caused by *Mesodinium rubrum* and *Gymnodinium catenatum* in northwest coast of Spain. J. Appl. Bacteriol. 68:123-132.

50. Sakata, T. 1990. Occurrence of marine Saprospira sp. possessing algicidal activity for diatoms. Nippon Suisan Gakkaishi 56:1165.

51. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning : a laboratory manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Habor, N. Y.

52. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

53. Seizen, R. J., and Leunisen, J. A. 1997. Subtiliases: the superfamily of subtilisin-like serine proteases. Protein Sci. 6:501-523.

54. Seizen, R. J., de Vos, W. M., Leunissen, J. A. M., and Dijkstra, B. W. 1991. Homology modeling and protein engineering strategy of subtiliases. The family of subtilisin-like serine proteases. Protein Eng. 4:719-737.

55. Shilo, M. 1970. Lysis of blue-green algae by *Myxobacter*. J. Bacteriol. 104:453-461.

56. Shimoi, H., and Tadenuma, M. 1991. Characterization of *Rarobacter faecitabidus* protease I, a yeast-lytic serine protease having mannose binding Activity. J. Biochem. (Tokyo) 110:608-613.

57. Shiomoi, H., Iimura, Y., Obata, T., and Tadenuma, M. 1992. Molecular structure of *Rarobacter faecitabidus* protease I: a yeast-lytic serine protease having mannose-binding activity. J. Biol. Chem. 267:25189-25195.

58. Shumway, S. E. 1990. A review of the effects of algal blooms on shellfish and

awuaculture. J. World Aquacult. Soc. 21:65-104.

59. Siezen, R. J., de Vos, W. M., Leunissen, J. A., and Dijkstra, B. W. 1991. Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Eng. 4:719-737.

60. Smith, P. K., Krohn, I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.

61. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

62. Staffan, Kjelleberg. 1999. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents Carola Holmstrom. MiniReview FEMS Microbiol. Ecol. 30:285-293.

63. Stewart, JR., Brown, RM. 1969. Cytophaga that kills or lyses algae. Science 164:1523-1524.

64. Stewart, WDP., Draft, MJ. 1976. Algal lysing agents of freshwater habitats. In: Skinner FA, Carr JG (Eds) Microbiology in agriculture, fisheries and food. Academic Press, London, pp. 63-90.

65. Suda, H., Aoyagi, T., Hamada, M., Takeuchi, T., and Umezawa, H. 1972. Antipain, a new protease inhibitor isolated from *Actinomycetes*. J. Antibiot. (Tokyo) 25:263-266.

66. Techkarnjanaruk, S., Pongpattanakitshote, S., and Goodman, A. E. 1997. Use of a promoterless *lazZ* gene insertion to investigate chitinase gene expression in the marine bacterium *Pseudoalteromonas* sp. strain S9. Appl. Environ. Microbiol. 63:2989-2996.

67. Thayer, M. M., Flaherty, K. M., and McKay, D. B. 1991. Three-dimensional structure of the elastase of *Pseudomonas aeruginosa* at 1.5-Å resolution. J. Biol. Chem. 266:2864-2871.

68. Tsujibo, H., Miyamoto, K., Tanaka, K., Kaidzu, Y., Imada, C., Okami, Y., and Inamori, Y. 1996. Cloning and sequence analysis of a protease-encoding gene from the marine bacterium *Alteromonas* sp. strain O-7. Biosci. Biotechnol. Biochem. 60:1284-1288.

69. Tsujibo, H., Toshida, Y., Imada, C., Okami, Y., Miyamoto, K., and Inamori, Y. 1991. Isolation and characterization of a chitin degrading marine bacterium belonging to the genus *Alteromonas*. Nippon Suisan Gakkaishi 57:2127-2131.

70. Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods in Enzymology 153:3-11.

71. Vieira, J., and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.

72. Yamamoto, Y., Nishizawa, S., Kuroda, N., Sakamoto, M. 1993. Occurrence of heterotrophic bacteria causing lysis of cynobacteria in a eutrophic lake. Jpn J. Phycol. 41:215-220.

73. Ymamoto, Y., Suzuki, K. 1990. Distribution and algal-lysing activity of fruiting *Myxobacteria* in Lake Suwa. J. Phycol. 26:45-51.

74. Yoshinaga, I., Kawai, T., Takeuchi, T., Ishida, Y. 1995. Distribution and fluctuation of bacteria inhibiting the growth of a marine red tide phytoplankton *Cymnodinium mikimotoi* in Tanabe bay (Wakayama Pref. Japan). Fish Sci. 61:780-786.

75. Yoshinaga, I., Kim, M., Katanozaka, N., Imai, I., Uchida, A., and Ishida, Y. 1998. Population structure of algicidal marine bacteria targeting the red tide-forming alga *Heterosigma akashiwo* (Raphidophyceae), determined by restriction fragment length polymorphism analysis of the bacterial 16S ribosomal RNA genes. Marine Ecology Progress series 170:32-44.

76. Yoshinaga, I., Kawai, T., and Ishida, Y. 1997. Analysis of algicidal ranges of the bacteria killing the marine dinoflagellate *Gymnodium mikimotoi* isolated from

Tanabe Bay, Wakayama Pref., Japan. Fish. Sci. 63:94-98.