Interspecies interaction stimulates gliding motility of filamentous photosynthetic bacteria through the action of protease

A doctoral dissertation

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Contents

Abstract

The thermophilic photosynthetic bacterium *Chloroflexus aggregans* has been widely found in microbial mats of hot springs all over Japan. *C. aggregans* has been reported as a major component in the microbial mats developed at the temperature of 50 to 70°C. *C. aggregans* shows gliding motility to form dense cell aggregation in a liquid medium, and the ability would contribute to the formation of microbial mats in natural hot springs. Interspecies interaction may occur in the microbial mats where bacterial cells of diverse species are densely packed. It has been reported that interspecies interaction causes various phenotypical changes including biofilm formation and motility. I studied the effect of coexisting bacteria on the cell aggregation of *C. aggregans*.

Heterotrophic bacteria were isolated from the microbial mats to evaluate effect on the cell aggregation of *C. aggregans*. Cell aggregation was promoted by the addition of culture supernatants of 5 isolates. Strain BL55a showed the most remarkable promoting effect on the cell aggregation and was revealed to be closely related to *Bacillus licheniformis* based on 16S rRNA gene sequences analysis. The promoting effect was completely suppressed after heating the culture supernatants at 105°C for 10 min. Size fractionation of the culture supernatant indicated that molecular weight of the promoting factor was above 10,000. From these results, a possible promoting factor was

extracellular enzyme. Protease activity was detected in all of the isolates that showed the promoting effect on cell aggregation. A purified protease obtained from *B.licheniformis* was also showed the promoting effect. These results indicate that protease in the culture supernatant promoted cell aggregation of *C. aggregans*.

To examine the possible digestion of *C. aggregans* cells by the heterotrophic bacteria, Strain BL55a was spread on agar medium that contains *C. aggregans* without any other carbon source. After 2 days of cultivation, colonies of BL55a were detected and cell lysis of *C. aggregans* around the colonies was observed. These results indicate that of *C. aggregans* were digested and prayed cells by BL55a.

Escape behavior of *C. aggregans* from protease was evaluated. *C. aggregans* cell-containing agar was placed between protease containing agar and fresh agar in a glass cuvette. During the incubation at 55°C in the light, cells of *C. aggregans* were moved to the fresh agar layer as if it had escaped from the attack of protease.

This is the first report that protease produced by other bacterium stimulates bacterial cell aggregation. Strain BL55a shows predatory behavior through protease, and *C. aggregans* moves away from the protease. Such escape behavior of prey from predator observed in this study has never been reported in bacteria.

General introduction

A filamentous photosynthetic bacterium *Chloroflexus aggregans*

The phylum *Chloroflexi* is a deep branching lineage of bacteria (Hanada & Pierson, 2006). The genus *Chloroflexus* includes two species, *C. aggregans* and *C. aurantiacus* (Pierson & Castenholz 1974; Hanada et al., 1995a). The two species in the genus *Chloroflexus* are thermophiles and exhibit multicellular filamentous morphology, anoxygenic photosynthetic ability and gliding motility (Hanada & Pierson, 2006). Slightly alkaline hot springs are one of the major habitats of *Chloroflexus* (Hanada & Pierson, 2006, Hanada et al., 1995b).

C. aggregans MD-66^T is the first isolate in this species obtained from Okukinu Meotobuchi hot springs in Japan (Hanada et al., 1995a). *C. aggregans* MD-66^T has multicellular filamentous morphology with 200-300 μm in length and shows gliding motility at 55°C (Hanada et al., 1995a). It has been reported that the gliding rates of *C. aggregans* was typically 1−3 μm per sec and 100 times faster than that of closely related species, *C. aurantiacus* (Hanada et al., 2002).

C. aggregans can form cell aggregates in a liquid medium. This cell aggregate is not firmly packed and is easily dispersed and indicating that extracellular adhesive materials are not a crucial factor in the aggregation (Hanada et al., 2002). In addition, the cell

aggregation is reversibly formed after dispersing cells under light conditions but not formed under anaerobic dark condition indicating the cell aggregation needs energy supply by photosynthesis and/or respiration (Hanada et al., 2002). These observations suggest that the cell aggregation is formed by their rapid gliding motility. Such an active cell aggregation is not observed in *C. aurantiacus* (Hanada et al., 2002). The rapid gliding motility and active cell aggregation are remarkable features of *C. aggregans*.

Gliding motility in bacteria

Gliding motility is defined as the movement in the direction of its long axis on a surface (Spormann, 1999). Many species of gliding bacteria have been isolated from various environments and are widespread into 10 phyla (Table 1). For the mechanisms of gliding motility, three different models have been proposed, i.e., a twitching motility, a helical loop track model and a centipede model.

Twitching motility depends on type IV pili (Wall & Kaiser 1999). The type IV pili are extracellular filamentous polymeric structures and located on pole of the cell body (Wall & Kaiser 1999). Cells extend the type IV pili and attach them to solid surfaces, and then retraction of the pilus results in movement of the cell. A centipede model is based on the four different functional proteins; a “leg” protein, “gear” protein, “motor”

protein and “mount” protein. The legs change ultra-structure and repeat “catch and release” the solid surface resulting in walking movements. In a helical loop track model, directed movement is achieved by cell rotation. The cell rotation is caused by adhesive proteins that travel along a helical loop on the cell surface.

No gliding mechanism has yet been proposed for bacteria in the phylum *Chlroflexi*. In *C. aggregans*, extracellular structures that observed in other gliding bacteria for the gliding motility have not been found (Hanada et al., 1995a). Genome analysis of *C. aggregans* indicated that they did not possess genes for gliding motility reported in other gliding bacteria, excepting for a part of possible genes related to twitching motility (e.g., *pilT* and *pilA*) (Klatt et al., 2007). These indicate that gliding mechanism of *C. aggregans* is different from those of other gliding bacteria.

Role of motility as a survival strategy in bacteria

Bacterial cellular motility likely plays a role in the adaptation to environments. Motile bacteria sense environmental factors such as chemicals, and change their motility. This behavior has been known as a chemotaxis (Larsen et al., 1974). Chemotactic bacteria detect chemical stimulus with chemoreceptors and regulate direction of cellular motility with a two-component signal transduction system (Grebe & Stock, 1998). The

chemotactic system enables bacterial cells to move towards to chemoattractants and away from chemorepellents. Interaction between microbial cells, either intraspecies or interspecies, may be related to cellular motility, but little is known about the effect of other bacteria on the bacterial motility.

Interspecies interactions in bacteria

Bacterial cell to cell communication has been accepted as a new field of microbiology in the past two decades. Studies of interspecies interactions are required for understanding microbial behavior in natural environments.

Extracellular signaling molecules inducing interspecies interactions have been identified; autoinducer-2, a class of modified furan, was reported as one of the common signal molecules in bacteria (Chen et al., 2002, Federle & Bassler, 2003). The study of autoinducer-2 started from the findings that luciferase synthesis of *Vibrio harveyi* was promoted by cell free culture supernatants of other bacteria (Greenberg et al., 1979). Federle and Bassler showed that over 50 species of bacteria including gram-positive and gram-negative bacteria possess the gene for specific receptor of autoinducer-2 by genome analyses (Federle & Bassler, 2003). Recent studies also revealed that interspecies interactions often occur via known molecules, such as antibiotics and a portion of cell wall (Shank & Kolter, 2009); antibiotics tetracycline and tobramycin

stimulated biofilm formation of *Pseudomonas aeruginosa* (Linares et al, 2006); disaccharide tripeptides containing diamonopimelic acid stimulated germination of *Bacillus subtilis* (Shah et al., 2008).

C. aggregans and co-existing bacteria in the microbial mats

C. aggregans-containing microbial mats are found in hot spring waters at 48−65°C in Nakabusa hot springs. In the mats, *C. aggregans* works as one of primary producers and co-exists with aerobic sulfide oxidizer, sulfur- and sulfate-reducers, cyanobacteria, fermenters and aerobic heterotrophs (Nakagawa & Fukui, 2002; Kubo et al., 2011; Everroad et al., 2012; Otaki et al., 2012). Otaki et al. reported that dense packing of cells in the microbial mats effectively promoted interspecies interaction through exchange their metabolites (Otaki et al., 2012).

In this study, I focused on interspecies interaction affecting cellular motility of *C. aggregans*. Aggregating ability and directional motility of *C. aggregans* cells are thought to be influenced by co-existing bacteria in the densely packed microbial mats. I explored heterotrophic bacteria which affected on the aggregating motility of *C. aggregans* from the microbial mats and tried to identify the interspecies interaction.

These are novel trials which explore interspecies interaction in thermophilic bacteria and cell-to-cell communication affecting the cellular motility.

Table 1. A list of gliding bacteria and expected mechanisms.

Phylum	Representative species	Expected gliding mechanism	Refences
Aquificae	*Aquifex aeolicus*	twitching	Herdendorf et al., 2002
Chloroflexi	*Chloroflexus aurantiacus*	unknown	Pierson and Castenholz, 1974
Planctomycetes	*Isosphaera pallida*	twitching	Giovannoni et al., 1987
Cyanobacteria	*Synechocystis* sp.	twitching	Bhaya et al., 2001
Fusobacteria	*Fusobacterium nucleatum*	unknown	Abbott and Sudo, 1977
Firmicutes	*Clostridium perfringens*	twitching	Varga et al., 2006
Bacteroidetes	*Flavobacterium johnsonie*	helical loop track model	Nakane et al., 2013
Chlorobi	*Chloroherpeton thalassium*	unknown	Gibson et al., 1984
Proteobacteria	*Neisseria gonorrhoeae*	twitching	Merz et al., 2000
	Pseudomonas aeruginosa	twitching	Semmler et al., 1999
	Myxococcus xanthus	twitching and helical loop track model	Mauriello et al., 2010
Tenericutes	*Mycoplasma mobile*	centipede model	Miyata, 2008

Chapter 1

Secreted protease mediates interspecies interaction and promotes cell aggregation of a thermophilic bacterium *Chloroflexus aggregans*

Abstract

Interspecies interactions were studied in hot spring microbial mats where diverse species of bacterial cells are densely packed. The anoxygenic photosynthetic bacterium, *Chloroflexus aggregans* has been widely found in the microbial mats as a major component in terrestrial hot springs in Japan at the temperature from 50 to 70°C. *C. aggregans* shows cellular motility to form a microbial mat-like dense cell aggregate. The aggregating ability of *C. aggregans* was affected by another bacterial species, strain BL55a (related to *Bacillus licheniformis*) isolated from the microbial mats containing *C. aggregans*. Cell aggregation rate of *C. aggregans* was promoted by the addition of culture supernatants of strain BL55a. Similar effects were also detected from other bacterial isolates, specifically *Geobacillus* sp. and *Aeribacillus* sp. Protease activity was detected from the culture supernatants from all of these isolates. The promoting effect of strain BL55a was suppressed by a serine protease inhibitor, phenyl methyl sulfonyl fluoride. A purified serine protease, subtilisin obtained from *B. licheniformis*, showed a promoting effect on the cell aggregation. These results suggest that an extracellular protease, secreted from co-existing bacterial species promoted the aggregating motility of *C. aggregans*. This is the first report that exogenous protease affects bacterial cellular motility.

Introduction

Chloroflexus aggregans is a thermophilic anoxygenic photosynthetic bacterium belonging to the family *Chloroflexaceae*, which is characterized as unbranched multicellular filaments that possess gliding motility (Hanada et al., 1995a; Hanada & Pierson, 2006). Gliding motility is defined as the movement in the direction of its long axis on a surface (Spormann, 1999). *C. aggregans* MD-66^T, isolated from Okukinu Meotobuchi hot springs in Japan, has 200-300 μm in length of multicellular filament and rapid gliding motility (1−3 μm s^{-1}) at 55°C (Hanada et al., 1995a). It has been reported that the motility of *C. aggregans* MD-66^T was influenced by light intensity, temperature and pH of the medium (Hanada et al., 2002). Gliding motility on the cell surface of other filaments makes this bacterium form dense cell aggregates. This cell aggregate is not firmly packed and is easily dispersed indicating that extracellular adhesive materials are not a crucial factor in the aggregation (Hanada et al., 2002).

Microbial interspecies interactions cause various phenotypic outcomes including biofilm formation and cell motility (Federle & Bassler, 2003; Shank & Kolter, 2009). Most of these interactions occur through extracellular signal molecules such as bacteriocins and have been found in environments where cell density is high (Kreth et al, 2005; Broderick et al, 2006; Shank & Kolter, 2009). *Chloroflexus* is widely distributed

in terrestrial hot springs in the temperature range of 50 to 70°C (Hanada et al., 1995b; Pierson & Castenholz, 2001) and is frequently found in microbial mats where bacterial cells are densely packed and exist in close proximity to each other.

Molecular ecological studies of the microbial mats from Nakabusa hot springs in Japan indicated that *C. aggregans* is often the major constituent co-existing with sulfur oxidizing bacteria, cyanobacteria and aerobic and anaerobic heterotrophic bacteria in microbial mats found at 48 to 65°C (Nakagawa & Fukui, 2002; Kubo et al., 2011; Everroad et al., 2012; Otaki et al., 2012). In this study, I isolated bacteria from the microbial mats at Nakabusa hot springs that coexisted with *C. aggregans*, and explored the effect of culture supernatants from these isolates on the cell aggregation rate of *C. aggregans*.

Materials and methods

Isolation of *C. aggregans* from Nakabusa hot springs

Microbial mats that had developed in an outflow (65°C, pH 8.8) of hot spring water were collected from Nakabusa hot springs (36°23’15’’N, 137°45’00E’’E, 1,500 m elevation) in Japan. Cultivation of *C. aggregans* was performed according to the procedure reported by Hanada et al. 1995a. A piece of the mats was anaerobically cultivated in 30 mL of PE medium at 55°C under incandescent light (30 W m^{-2}). After a week, growth was observed and the culture was spread onto PE medium supplemented with 1.5% agar (Wako, Tokyo, Japan). The plate was incubated at 55°C under anaerobic conditions in the light. Anaerobic conditions were achieved by using the oxygen absorber (Ever-Fresh, Torishige Sangyo, Oita, Japan) in a sealed nylon bag. From these isolates, brown colored colonies were isolated. After 5 successive platings of colonies, a pure culture was established.

Isolation of bacteria coexisting with *C. aggregans* in the microbial mats

Microbial mats that had developed at either 55°C or 65°C at Nakabusa hot springs were collected. 0.1 g of the mats was homogenized and the suspension was spread on PE medium described above or on nutrient medium (Eiken Chemical Co., Tokyo, Japan)

supplemented with 1.5% agar. These plates were cultivated at 50°C under aerobic conditions. After 1 day of incubation, colonies were isolated and purified by repetitive cultivation using the same medium.

Analysis of 16S rRNA gene sequence of isolates

DNA of isolates was extracted as described previously (Stahl et al., 1988). Bacterial cells were disrupted by bead beating using 0.1 mm diameter zirconia-silica beads (Biospec Products, Bartlesville, OK, USA). Genomic DNA was purified with phenol extraction, chloroform-isoamyl alcohol extraction and ethanol precipitation.

The 16S rRNA gene of isolates was amplified using the 27F (AGAGTTTGATCMTGGCTCAG) and 907R (CCGTCAATTCMTTTGAGTTT) primers (Lane et al., 1985; Lane et al., 1991). PCR was performed with Ex-Taq polymerase (Takara, Otsu, Japan). PCR amplification was performed using a 2720 Thermal Cycler (Applied Biosystems) under the following conditions: initial denaturation at 94ºC for 3 min; 30 cycles of denaturation at 94ºC for 30 sec, primer annealing at 55ºC for 45 sec, and extension at 72ºC for 1 min; and a final elongation at 72ºC for 4 min. PCR products were sequenced using the Big Dye Terminator v3.1 Sequencing kit (Applied Biosystems) and on a ABI3130xl Genetic Analyzer (Applied

Biosystems). The sequences were analyzed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Measurement of protease activity

Protease activity was assayed using azocasein as the substrate as described by Secades et al., 1999. Bacterial isolates were aerobically cultured at 50°C in PE or nutrient liquid medium and the cultures at late exponential growth phase were centrifuged to collect the supernatants. 120 μL of the culture supernatant was mixed with 480 μL of 10 mM Tris-HCl pH 8.0 containing 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.2% azocasein (Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 30 min at 55°C. The reaction was stopped by adding 600 μL of 10% trichloroacetic acid. The tubes were centrifuged at 12,000 x *g* for 20 min and 800 μL of the supernatant was collected. Then 200 μL of 2 N NaOH was added to the collected supernatant. The release of azo dye from azocasein was measured at 440 nm using an Infinite 200 PRO microplate reader (Tecan, Seestrasse, Switzerland). One unit of protease activity was defined as the amount that increased the A_{440} by 0.1 in 30min.

Cell aggregation test

C. aggregans isolated in this study was anaerobically grown in PE medium in the light. One mL of the culture collected at exponential phase of growth was mixed with 6 mL of fresh PE medium in a glass test tube (1.5 cm diameter × 9 cm height). The cell suspension was incubated at 55°C in the light (30 W m^{-2}) to form cell aggregates from the dispersed cells in a tube.

To evaluate potential chemical activities from other bacteria on the aggregation of *C. aggregans*, bacterial isolates were cultivated in liquid medium as described above. Culture supernatants from these cultures were obtained by filtration through a membrane filter (pore size = 0.22 μm). Six mL of the culture supernatant was mixed with one mL of the *C. aggregans* culture and incubated as described above to determine the effect on the aggregation. As a control, fresh medium that was used for cultivation of isolates was selected instead of the culture supernatants.

In order to estimate the effective chemical species in the culture supernatants, a series of parallel experiments was performed assuming that it may be a protein: (a) the culture supernatant was incubated at 105°C for 10 min before mixing with the *C. aggregans* culture; (b) fractionation of the culture supernatants were conducted; different size fractions were provided to *C. aggregans* cultures. These fractionations

were conducted as follows; 10 mL of the bacterial culture supernatants were applied to Amicon Ultra Centrifugal Filters (molecular cut off = 10 kDa, Millipore, Billerica, USA) to obtain concentrate [high-molecular weight (HMW) fraction, M.W. > 10,000] and filtrate. The filtrate, which mainly contained the low-molecular weight fraction, was filled to 10 mL with fresh PE medium (LMW fraction, M.W. < 10,000); (c) the effect of serine protease inhibitor was determined; phenyl-methyl-sulfonyl-fluoride (PMSF, Sigma, Saint Louis, USA) was added to the culture supernatants (final concentration, 1 mM) and incubated at room temperature for 10 min before the cell aggregation tests.

A commercially available protease (protease from *Bacillus licheniformis*, Sigma, Saint Louis, USA) was used to evaluate the effect of protease on the cell aggregation. Its protease activity was determined as described above and the protease was diluted with PE medium to obtain the appropriate amount of activity before testing.

Characterization of compounds released from protease-treated *C. aggregans* cells

C. aggregans was aerobically cultivated in 20 mL of PE medium. Cells collected at exponential growth phase were washed 3 times with 10 mM Tris-HCl pH 8.0. The washed cells were resuspended in 20 mL of 10 mM Tris-HCl pH 8.0 containing 10 U

mL^{-1} of the purified protease from *B. licheniformis* (Sigma, Saint Louis, USA) and incubated for 30 minutes at 55°C. After that, PMSF was added into the reaction mixture (final concentration, 1 mM) to stop the reaction. Cells were removed from the reaction mixture by centrifugation (7,096 × g, 10 min) and filtration through a membrane filter (pore size = 0.22 μm). High molecular weight compounds including the protease were removed by ultrafiltration (Amicon Ultra Centrifugal Filters, molecular cut off = 10 kDa, Millipore, Billerica, USA) to collect low molecular weight (LMW) fraction.

In order to characterize the effective chemical species in the LWM fraction, a series of parallel experiments was performed assuming that it may be a peptide: (a) the LWM fraction was heated at 98°C for 10 min; (b) In order to estimate the molecular weight of the effective chemicals, size fractionation and polyacrylamide gel electrophoresis were conducted. Cells collected from 1 L of culture solution were treated with the protease (10 U mL^{-1}) in 10 mM Tris-HCl pH 8.0 for 60 minutes at 55°C. After removal of the cells by centrifugation and filtration through a membrane filter (pore size = 0.22 μm), the supernatant was applied to PD-10 column (GE Healthcare UK Ltd., Buckinghamshire, UK). The 10 μL of each fraction was serially collected and the cell aggregation promoting activities were examined for 100 μL the culture solution at exponential phase of growth. The active fractions were analyzed by Tricine-SDS-PAGE

on a 16% polyacrylamide gel and visualized by silver staining. Silver staining was performed using Silver Stain Kit Wako (Wako, Tokyo, Japan).

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences obtained in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases. The accession number of strains NBF, BL55a, MS01, KS001, KS02, KS03 and KS04 are respectively LC003597 -LC003603.

Results

Cell aggregation of *C. aggregans* isolate obtained from Nakabusa hot springs

C. aggregans strain NBF isolated from the microbial mats at Nakabusa hot springs in Japan shared 98.7% identity with that of *C. aggregans* MD-66^T (Hanada et al., 1995a) in the 16S rRNA gene sequences. Cell aggregation of strain NBF was observed at 55°C in the light as reported for the type strain MD-66^T. As shown in Fig. 1-1, the dispersed olive-greenish-cells in the medium in 5.5 cm height gradually aggregated at the bottom of the tube. These aggregations were not observed in the dark. Time for formation of the cell aggregates below 2.5 cm from the bottom of the tube (Fig. 1-1) was used for the index of the aggregation in this paper.

Effect of culture supernatant on the cell aggregation of *C. aggregans*

Six strains of heterotrophic aerobes were isolated from the microbial mats. Phylogenetic analysis based on partial 16S rRNA gene sequences indicated these isolates belonged to the genus *Bacillus*, *Meiothermus*, *Geobacillus*, or *Aeribacillus* (Table 1-1). Culture supernatants of these isolates were prepared to evaluate effect on the cell aggregation of *C. aggregans* strain NBF. Cell aggregation was promoted by the

addition of culture supernatants from *Bacillus* sp. strain BL55a (Table 1-1); addition of BL55a culture supernatant shortened the time for formation of the cell aggregates 1.9 fold. A little less promoting effects, 1.7, 1.4 and 1.5 fold, were also observed for *Geobacillus* sp. strain KS02 and *Aeribacillus* sp. strains KS03 and KS04, respectively. The culture supernatant of *Meiothermus* sp. strain MS01 showed no effect on the cell aggregation.

Characterization of the aggregation promoting factor

Bacillus sp. strain BL55a showed the largest promoting effect on the cell aggregation of *C. aggregans* strain NBF. The promoting effect by strain BL55a was examined under various conditions (Fig. 1-2). The time required for formation of 2.5 cm height aggregates was about 40 min when the culture supernatant of strain BL55a was added, although it took longer than 80 min without the culture supernatant (Fig. 1-2, bars 1 and 2). This promoting effect of the culture supernatant was completely suppressed after heating the culture supernatant at 105°C for 10 min (Fig. 1-2, bar 3).

Size fractionation by ultrafiltration was performed to estimate the molecular weight of the promoting factor. LMW fraction of the culture supernatant, obtained through the filter of molecular weight cut-off 10,000 did not promote the cell aggregation rate (Fig.

1-2, bar 4). Conversely, the HMW fraction, i.e., M.W. > 10,000, shorten the time to about 48 min (Fig. 1-2, bar 5).

Effect of protease on the cell aggregation of *C. aggregans*

I thought that a possible promoting factor in the culture supernatant was extracellular enzyme, e.g., protease, since thermophilic bacilli have been known to secrete serine proteases (Ferrero et al., 1996; Cihan et al., 2011). In fact, protease activity was detected from all of the isolates that showed the promoting effect on cell aggregation of *C. aggregans* strain NBF (Table 1-1), but was hardly detected from strain MS01. The promoting effect of the supernatant from *Bacillus* sp. strain BL55a was suppressed by the addition of PMSF, a serine protease inhibitor, while PMSF added alone did not affect the cell aggregation rate (Fig. 1-2, bars 6 and 7).

A serine protease, subtilisin prepared from *Bacillus licheniformis* is commercially available and its effect on the cell aggregation of *C. aggregans* strain NBF was evaluated. The promoting ability of this subtilisin was observed when the activity of the purified protease, i.e., 7 U mL^{-1} was comparable to that of the culture supernatant of strain BL55a (Fig. 1-2, bars 8 and 2), however excess amount of protease caused cell lysis (data not shown).

The different concentration of protease was prepared to evaluate effect on the cell aggregation and growth. The cell aggregation was promoted when protease concentration was higher than 1 U mL^{-1} (Fig. 1-3 A). The protease was dose-dependently promoted the cell aggregation. Each test tube was incubated at 55°C in the light after the cell aggregation tests in succession. Fig. 1-3B shows growth curves of *C. aggregans* in the presence of protease. Growth inhibition was observed when protease concentration was 1 U mL^{-1}, while 0.1 U mL^{-1} of protease did not affect the growth. Initial cell density was decreased under 10 and 100 U mL^{-1} of protease. These results mean cell lysis of *C. aggregans* was occurred under high concentration of protease. Concentrations of the protease effective for the promotion of cell aggregation were correlated with the concentrations which suppress the growth.

Effect of compounds released from protease-treated *C. aggregans* cells on the cell aggregation

I hypothesized that the cell surface protein of *C. aggregans* was degraded by protease and the degradation products promoted cell aggregation. To obtain the degradation products, *C. aggregans* cells were reacted with 10 U mL^{-1} of protease in 10 mM Tris-HCl pH 8.0 for 30 minutes. After removal of cells from the solution, low

molecular weight fraction was obtained through the filter of molecular weight cut-off 10,000 to remove protease. This fraction showed the promoting activity of the cell aggregation (Fig. 1-4). The promoting activity of the fraction was stable to heat treatment at 98°C for 10 min. Gel filtration was conducted to achieve size fractionation and the active fractions were collected (Fig. 1-5A). SDS-PAGE analysis showed that active fractions contained peptide bands with around M.W. 3,000 (Fig. 1-5B).

Discussion

In this work, I isolated 6 strains of heterotrophic bacteria co-existing with *C. aggregans* in microbial mats. Most of these bacterial isolates showed the promoting effect on the cell aggregation of *C. aggregans* strain NBF (Table1-1). From the results shown in Fig. 1-2, I concluded that protease accelerated the cell aggregation rate of *C. aggregans*.

Connelly et al. 2004 reported that *Bacillus subtilis* required its own extracellular protease for swarming motility. They suggested that peptides produced by proteases may work as signal molecules for motility. Cyclic- or linear-peptides have been known to act in cell-cell communication in gram-positive bacteria (Schauder & Bassler, 2001; Yeo et al., 2012). In this study, products containing peptide derived from protease-treated cells promoted cell aggregation of *C. aggregans*. The peptides may work as signal molecules and cause promotion of the cell aggregation.

Growth inhibition or cell lysis of *C. aggregans* strain NBF was observed when protease concentration was sufficient for the promotion of cell aggregation (Fig. 1-3). Production of proteases may be a predatory strategy of heterotrophic bacteria that allows them to prey cells to feed upon primary producers such as *C. aggregans*. Destruction of prey cells to feed upon is one of the most common behaviors in

predatory bacteria (Jurkevitch, 2007). Protection from bactericidal reagents by formation of cell aggregates or biofilms is known for a wide variety of bacteria (Høiby et al., 2010). My findings in this study suggest a protease-producing predator-prey interaction; however, rapid formation of cell aggregates of a pray, *C. aggregans* may reduce the predation pressure.

This is the first report that the directed motility toward aggregation of bacteria is promoted by extracellular proteases produced by other bacteria, but the molecular mechanisms of this behavior still remain to be elucidated. However, the interspecies interaction observed here does not seem to be highly specific. I speculate that signal transduction induced by extracellular proteases or their peptide products is widely distributed in nature and these relationships may sometimes be responsible for the development and maintenance of microbial consortia.

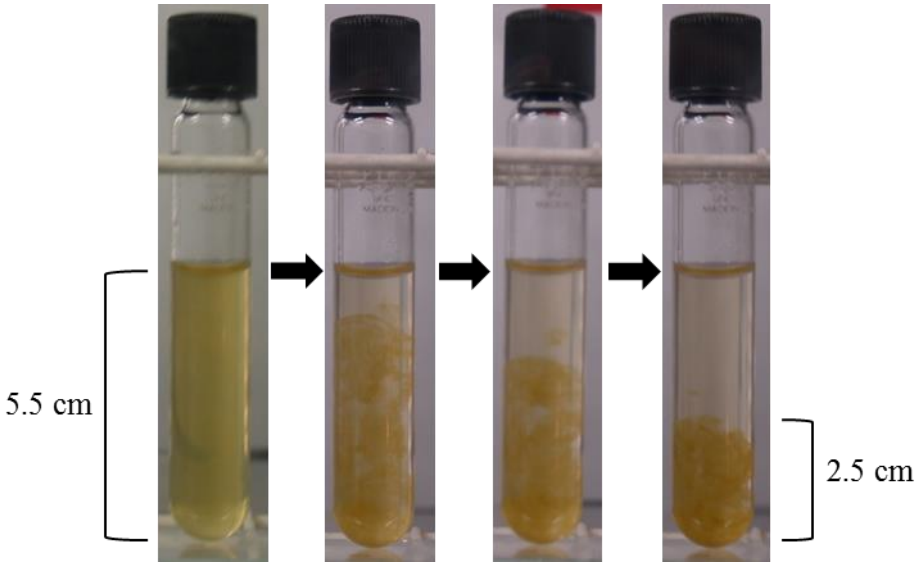


Fig.1-1. Cell aggregation of *C. aggregans* strain NBF in a glass test tube.

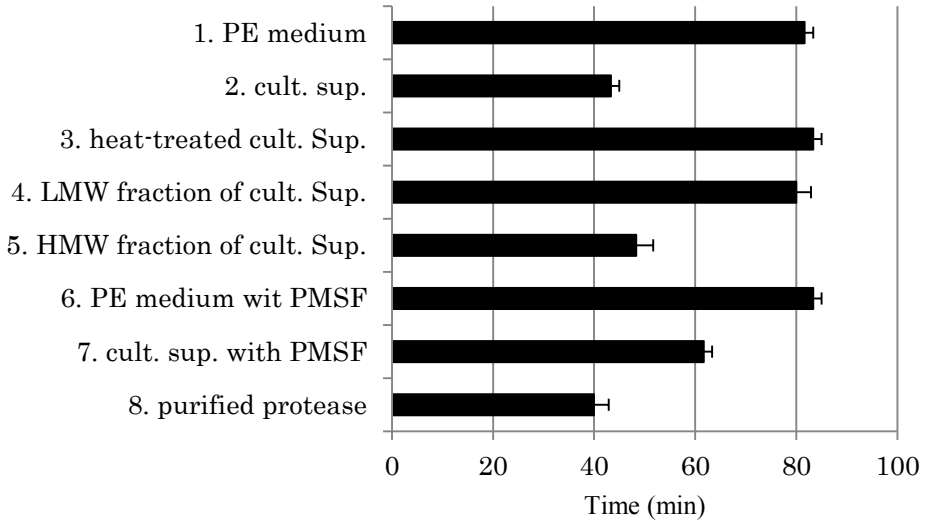
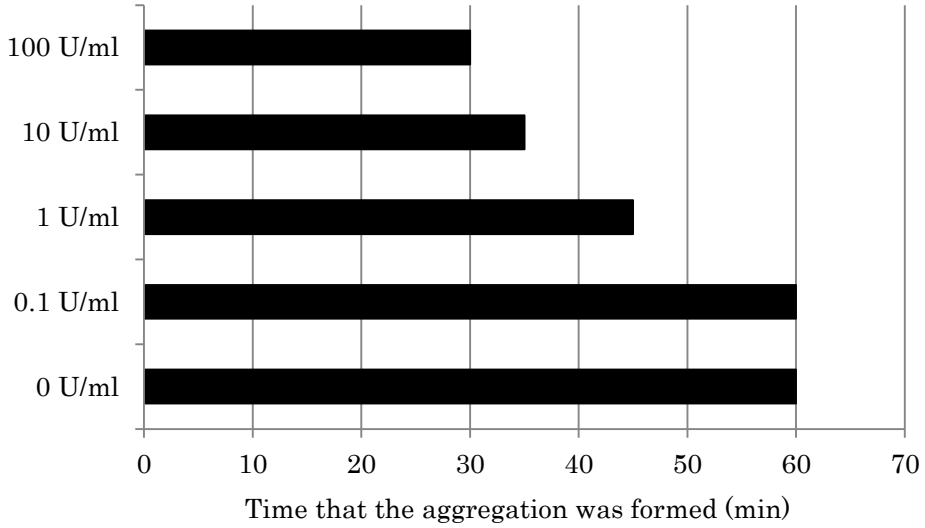


Fig. 1-2. Effect of culture supernatants of strain BL55a (cult. sup.) on the cell aggregation rate of *C. aggregans* in the test tube.

The culture of *C. aggregans* in PE medium was mixed with the following solution; 1, fresh PE medium; 2, culture supernant of strain BL55a (cult. sup.); 3, heat-treated cult. sup. (105°C, 10 min); 4, LMW fraction (M.W. < 10,000) of cult. sup.; 5, HMW fraction (M.W. > 10,000) of cult. sup.; 6, PE medium containing PMSF; 7, cult. sup. containing PMSF; 8, purified protease in the PE medium (final conc., 7 U mL^{-1}). The cell suspension was incubated in the test tube at 55°C in the light. Time for formation of the cell aggregates with 2.5 cm height (as shown in Fig. 1-1) was measured. Error bars indicate SD (n=3).

A



B

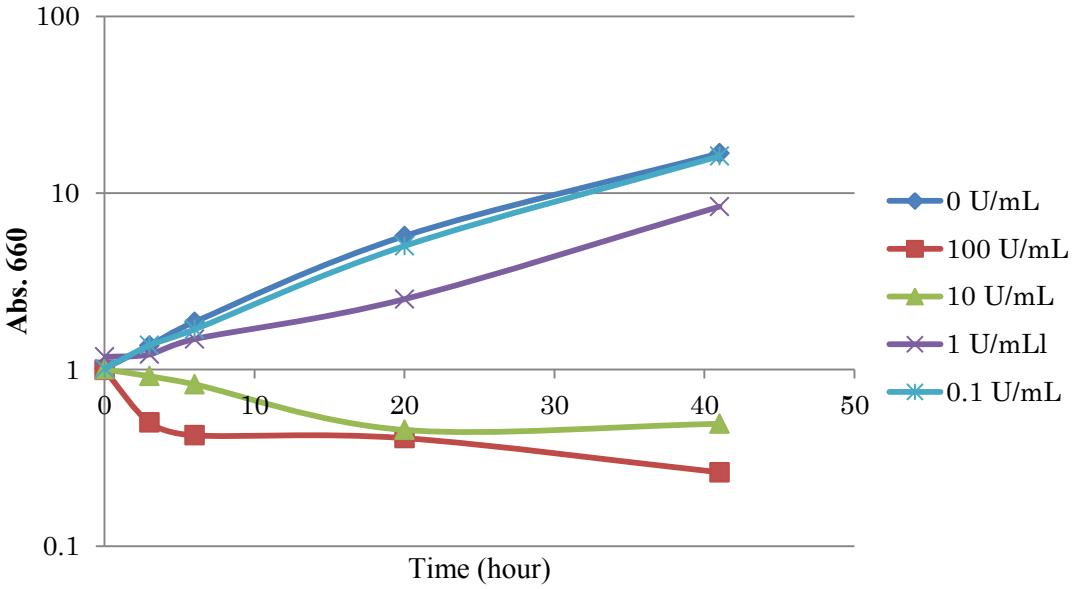


Fig. 1-3. Relations between the promoting activity and growth.

(A) Effect of protease concentration on the promoting activity. (B) Growth of *C. aggregans* in the presence of difference amount of protease. After testing the promoting activity for (A), these tubes were cultivated to observe their growth (B).

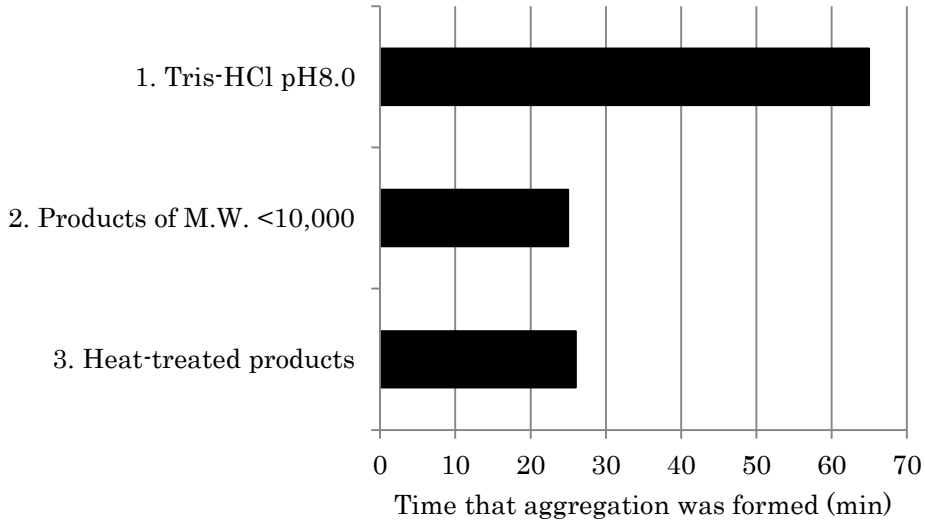
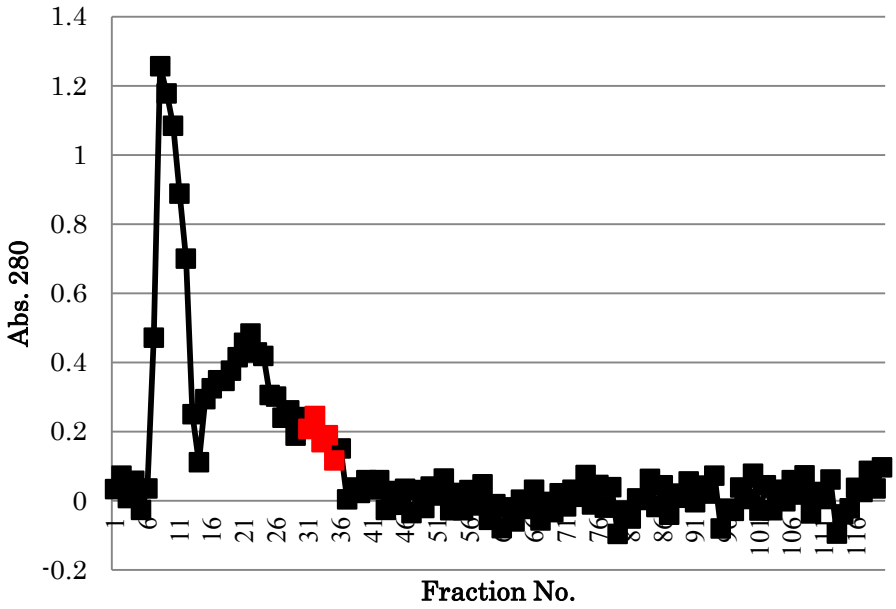


Fig. 1-4. Effect of compounds released from protease-treated *C. aggregans* cells on the cell aggregation of *C.aggregans*.

The culture of *C. aggregans* in 10 mM Tris-HCl pH 8.0 was mixed with the following solution; 1, 10 mM Tris-HCl pH8.0; 2, M.W. < 10,000 fraction of protease-cells treated products; 3, heat-treated M.W. < 10,000 fraction of protease-cells treated products (98°C, 10 min).

A



B

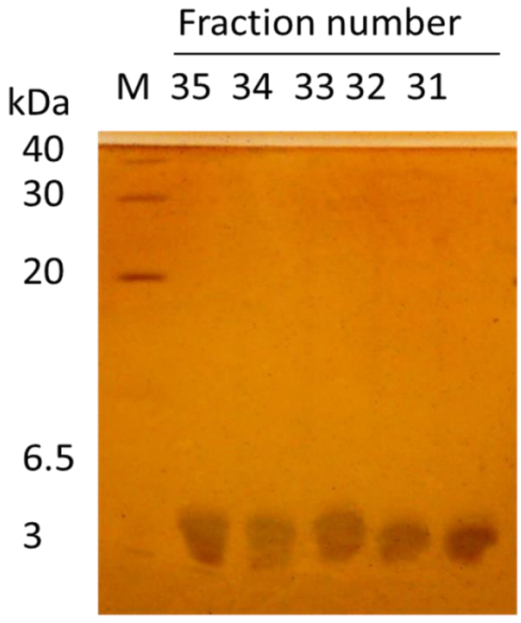


Fig. 1-5. Molecular weight of the promoting factor in the degradation products.

(A) Size fractionation of the degradation products using PD-10. Red squares mean the active fractions. (B) SDS-PAGE and silver staining of the active fractions.

Table 1-1. A list of strains from hot spring microbial mats isolated in this study.

Strains	**Temperature of sampling site (°C)**	**Closest relatives (identity %, 16S rRNA gene seq.)**	**Promoting effect (fold)***	**Protease activity (U mL^{-1})****
BL55a	55	*Bacillus licheniformis* strain GBPI47 (100)	1.9 ± 0.04	7.5 ± 0.2
MM	55	*Meiothermus cateniformans* strain L462 (100)	1.0 ± 0.02	0.3 ± 0.1
KS01	65	*Aeribacillus palidus* strain GS3372 (100)	1.4 ± 0.04	8.0 ± 1.0
KS02	65	*Geobacillus stearothermophilus* strain Da7 (100)	1.7 ± 0.13	6.6 ± 0.3
KS03	65	*Aeribacillus palidus* strain GS3372 (99.9)	1.4 ± 0.16	6.6 ± 0.2
KS04	65	*Aeribacillus palidus* strain GS3372 (99.9)	1.5 ± 0.09	9.1 ± 0.3

*Promoting effect was calculated by dividing the times required for the formation of 2.5 cm height aggregates of *C. aggregans* in PE medium by the times in the medium mixed with the culture supernatant of isolates. Each value is the mean ± SD of three trials.
**Protease activity of the culture supernatant was determined using azocazein as the substrate. One unit of the activity was defined as the amount that increased the A_{440} by 0.1 at 55°C in 30 min.

Chapter 2

Escape behavior of *Chloroflexus aggregans* from protease-producing bacteria

Abstract

Protease-producing bacteria had been isolated from microbial mats in Nakabusa hot springs in Japan. In this chapter, I examined predator-prey interaction between a protease-producing bacterium, *Bacillus* sp. strain BL55a and *C. aggregans*. Growth of strain BL55a on agar medium that contains *C. aggregans* as a sole carbon source was observed. Strain BL55a formed colonies on the medium and clear zones around the colonies were observed. These results indicate degradation and utilization of *C. aggregans* cells by strain BL55a. Escape behavior of *C. aggregans* from protease was evaluated. *C. aggregans* cells were suspended into agar medium and solidified. The cells containing agar was placed next to the agar medium containing protease. During the incubation at 55°C, *C. aggregans* population moved away from protease although a part of cells were degraded. These results suggest that protease caused negative chemotactic behavior of *C. aggregans*. These results indicate that *C. aggregans* escape from predation through the action of protease. This is the first demonstration that bacteria response and change their behaviors via extracellular enzymes secreted from other bacteria.

Introduction

It has been known that bacteria form communities with a variety of species of bacteria in natural environments. In such communities, cooperative interaction through intracellular metabolites has been found (e.g., Otaki et al., 2012). Competitive interaction has been also known; predation is one of the most offensive behaviors of the competitive interaction. Predatory bacteria have been found in 15 families in 5 phyla (Jurkevitch & Davidov 2006; Jurkevitch 2007). Well-known predatory bacteria, the genus *Bdellovibrio* and its related bacteria, can grow only by feeding on other bacterial cells directly. Most of other predators produce cell-lytic enzymes and feed on the cellular materials (Jurkevitch & Davidov 2006; Jurkevitch 2007).

Although predatory behaviors of prokaryotes have been addressed in various reports, studies on defense mechanisms against prokaryotic predator are limited. Recently Muller et al. reported that production of an antibiotic, bacillaen, and sporulation protected *Bacillus subtilis* from predation by *Myxococcus xanthus* (Müller et al., 2014). However, there is no report that shows escape behavior of prey with cellular motility from predator in bacterial world, even though motility is thought as a fundamental defense reaction of bacteria against repellents.

In chapter 1, I isolated the filamentous photosynthetic bacterium *C. aggregans* strain

NBF and *B. licheniformis* strain BL55a from microbial mats at Nakabusa hot springs in Japan. The cell aggregation rate of *C. aggregans* was promoted by extracellular protease secreted from *B. licheniformis*, while excess amount of the protease caused cell lysis of *C. aggregans*. These results led to examine predator-prey interactions between them. Here, I show that predatory behavior of *B. licheniformis* against *C. aggregans* and escape behavior of *C. aggregans* from the predation.

Materials and methods

Bacterial strains and growth conditions

Chloroflexus aggregans strain NBF was isolated from microbial mats in an outflow of hot spring water (65°C, pH 8.8) at Nakabusa hot springs (36°23’15’’N, 137°45’00E’’, 1,500 m elevation) in Japan (see Chapter 1). *C. aggregans* was anaerobically grown in PE medium in the light according to the procedure reported by Hanada et al. 1995a. *Bacillus licheniformis* strain BL55a isolated from the microbial mats at Nakabusa hot springs was aerobically cultivated in the nutrient medium (Eiken Chemical Co., Tokyo, Japan) at 55°C (see Chapter 1). *Aeribacillus* sp. strain NOT, which had been isolated from the microbial mats developed at 65°C at Nakabusa hot springs (Kato, S., personal communication), was also aerobically cultivated on the nutrient medium at 55°C.

Predation assay of *Bacillus* sp. strain BL55a

C. aggregans cells grown in PE medium under the light conditions were collected at exponential phase of growth and washed 3 times with PBS buffer. The washed cells were suspended into the sterilized AP medium supplemented with 1.5% agar at 55°C. AP medium contains (per liter) 0.5 g of $(NH_4)_2SO_4$, 5 mL of a 1 M phosphate buffer, 1 mL of a vitamin mixture (Hanada et al., 1995a), and 5 mL of a basal salt solution

(Hanada et al., 1995a). Cultures of *Bsacullus* sp. strain BL55a and *Aeribacillus* sp. strain NOT were streaked on the medium containing *C. aggregans* cells as a prey and cultivated at 55°C aerobically.

Effect of protease on the direction of movement of *C. aggregans* cellular population

300 μL of MA medium (see below) supplemented with 0.7% agar was poured into a glass cuvette (1 cm width × 0.5 cm depth × 5 cm height) and solidified (lower layer). *C. aggregans* cells harvested at exponential phase of growth were suspended with MA agar at 55°C. 400 μL of the cell suspension was overlaid on the lower layer in the cuvette and solidified (middle layer). A commercially available protease (Protease from *Bacillus licheniformis*, Sigma, Saint Louis, USA) was mixed with MA agar at 55°C (final concn. = 15 U mL^{-1}). 300 μL of the protease containing MA agar was overlaid on the middle layer in the cuvette (upper layer). The cuvette was capped by rubber stopper and the gas phase was replaced to N_2 gas. As a control, MA agar containing no protease was overlaid as the upper layer. The cuvette was incubated at 55 °C under incandescent light (30 W m^{-2}) or dark condition. MA medium (per liter) contains 0.05 g of yeast extract, 0.05 g of thiosulfate, 5 mL of a 1 M phosphate buffer, 1 mL of a vitamin mixture, and 5

mL of a basal salt solution.

Absorption spectrum of the cuvette was periodically recorded between 500 nm and 800 nm (UV1800, Shimadzu, Kyoto, Japan) during the incubation. Absorbance at 740 and 673 nm were used for index representing the amount of bacteriochlorophyll *c* and bacteriopheophytin respectively. To determine the absorption spectrum of 1 mm interval within the middle layer and the surrounding parts of the cuvette, the cuvette in the spectrophotometer was moved up and down using a self-made cuvette holder.

Measurement of gliding rates and frequency of change in direction

C. aggregans was photoheterotrophycally cultivated and cells were collected at exponential phase of growth. Cells were suspended in fresh PE medium and poured into a “tunnel glass slide” that described as Uenoyama et al. (2004). The glass slide was incubated for 10 minutes at 55°C to allow cells to attach the glass surface in the light. Gliding motility of individual cell was observed with Olympus CX41 or Zeiss AX10. Thermal conditions of the microscope stage were maintained with a glass heater S-101 and a thermal controller BT-102A (BLAST, Kanagawa, Japan). The non-attached cells were removed by a flow of appropriate amount of PE medium, and then PE medium in the glass slide was replaced with PE medium containing the appropriate amount of

protease (protease from *Bacillus licheniformis*, Sigma, Saint Louis, USA).

The gliding movement of 30 filaments was recorded by Olympus DP73 video camera to calculate the gliding rate for 30 sec. The gliding rate was calculated by cellSens standard imaging software (Olympus, Tokyo, Japan). The video image was recorded for 100 min using HC-V720M high definition video camera (Panasonic, Osaka, Japan). The reversal frequencies were counted for 10 min.

Results

Growth of protease-producing bacteria utilizing *C. aggregans* cells as nutrients

The protease producer, *Bacillus* sp. strain BL55a was streaked on the agar plates containing *C. aggregans* cells as a sole carbon source. After 1 day cultivation at 55°C under aerobic and light conditions, colonies of *B. licheniformis* strain BL55a appeared and cell lysis of *C. aggregans* around the colonies was observed (Fig. 2-1). *Aeribacillus*.sp. strain NOT, isolated from the same microbial mats did not grow on the agar plate and did not show the ability of cell lysis of *C. aggregans* (Fig. 2-1). Extracellular proteolytic activity was not detected from the culture supernatant of *Aeribacillus* sp. strain NOT (data not shown).

Escape behavior of *C. aggregans* cellular population from protease

Effect of protease on the movement of *C. aggregans* cellular population was observed in the cuvette containing three layered agar media; upper layer, protease; middle layer, *C. aggregans* cells; lower layer, fresh medium. During the cultivation at 55°C under anaerobic conditions, spatial distribution of *C. aggregans* was measured by spectrophotometer. Fig. 2-2A shows that cell density was obviously decreased at the

positions close to the protease layer (positions 4−8) and increased at the boundary between the *C. aggregans* layer and the fresh medium layer (positions 12 and 13) for three hours. These phenomena were repeatedly observed as shown in Fig. S1. Cell density was obviously decreased at the positions close to the protease layer (positions 2−8) and increased at the boundary between the *C. aggregans* layer and the fresh medium layer (positions 12 and 13) for three hours (Fig. S1-A). After 30 hours incubation, the accumulation of *C. aggregans* in the opposite direction of protease, i.e., the lower layer was visually observed (Fig. 2-2B). No dynamic changes in cell distribution occurred when the upper layer did not contain the protease (Fig. 2-3, Fig. S1-B).

Lysis of *C. aggregans* cells during incubation in the cuvette was evaluated by the spectroscopic detection of bacteriopheophytin that was derived from degradation process of bacteriochlorophyl *c* (BChl *c*). At the position 6 of the cuvette shown in Fig. 2-2 where marked decrease of Abs 740 was observer, absorption spectrum was determined. After 3 hours of incubation, an absorption peak was slightly detected at 672 nm corresponding to bacteriopheophytin although this peak of BChl *c* was obviously decreased (Fig. 2-4A). The peak was also confirmed in the second derivative spectrum (Fig. 2-4C). On the other hand, the peak of bacteriopheophytin did not appear in the

cuvette containing no protease (Fig. 2-4B). The second derivative values at 672 nm were determined throughout the positions 1-17 during the incubation. As shown in Fig. 2-5, decrease of the second derivative value was detected at the positions 5−8 in 3 hours. This result indicates that a part of decrease in the amount of Bchl *c* at the positions 4−8 in Fig. 2-2 was caused by degradation of cells by protease.

Microscopic observation of cellular motility of *C. aggregans* in the presence of protease

Effect of the protease on cellular motility of *C. aggregans* was examined with microscope. Firstly, gliding velocity of *C. aggregans* on a glass surface was measured in the presence or in the absence of protease. The video images were recorded for 30 seconds and the gliding rates of 30 filaments were calculated. As shown in Fig. 2-6, protease did not affect the gliding velocity, i.e., filamentous cells of *C. aggregans* in the growth medium in the absence of protease moved at a rate of 1.9 ± 0.34 μm sec^{-1} at 55°C and the rate in the presence of protease was 2.0 ± 0.35 μm sec^{-1}.

Next, frequency of change in the direction of motility was examined. The video images were recorded for 10 min and the reversal frequencies of 30 filaments were counted. *C. aggregans* cells moved in the direction of the long axis and turn the direction every 0.25 ± 0.18 minutes in PE medium containing no protease (Fig. 2-7).

The frequency was 0.55 ± 0.2 per min in the presence of protease.

Discussion

In this work, I found the prey-predator interaction between *C. aggregans* and protease-producing heterotrophic bacteria isolated from microbial mats. From the results shown in Fig. 2-2, I concluded that *C. aggregans* had the escaping motility away from protease.

Fig. 2-1 suggests that *Bacillus* sp. strain BL55a take up nutrients from *C. aggregans* cells. Extracellular protease produced by strain BL55a likely contributed to lyse the prey cells. It has been reported that bacteriolytic *Bacillus* species feed on other bacteria (Brack et al., 2013). Molecular ecological analyses by Everroad et al. indicated the existence of several species of protease producers in the microbial mats (Everroad et al., 2012). Three species of protease-producing heterotrophic bacteria have been isolated from the microbial mats (Table1-1). Using the same method shown in Fig. 2-1, I have also confirmed the predatory growth of these protease-producing bacterial isolates (data not shown). Predatory behavior by secretion of cell lytic enzymes may be a general strategy in the densely packed microbial communities.

It has been known that bacteria have a mechanism sensing external stresses and show the responses by changing gene expression (Hengge, 2011). Motility is one of the responding behaviors to environmental stresses, and known as negative taxis (Larsen et

al., 1974). As shown in Fig. 2-2, cellular population of *C. aggregans* moved in the opposite direction from protease. This result is the first demonstration that bacterial enzyme induces change in the direction of bacterial motility. This means the first finding of escape behavior from predation in bacteria. Protease is one of the major bacteriolytic enzymes of bacterial predators (Jurkevitch & Davidov 2006). The protease–mediated stress response may widely distribute in prey-predator interaction in bacteria.

Microscopic observation revealed that *C. aggregans* cells increased reversal frequency when they exposed to the protease. Such response has been found in chemotactic bacteria (Larsen et al., 1974). Swimming bacteria (e.g., *Escherichia coli*) exhibit straight line motility and erratic motion called tumbling. The tumbling causes random changes in the direction. Frequency of the tumbling is controlled according to the concentration of chemo-repellent/attractant. When cells expose to high concentration of repellents, cells increase the tumbling/reversal frequency to look for new places randomly. If they find the place where the concentration of repellents is low, tumbling/reversal frequency is decreased, i.e., the direction is fixed. This behavior results in some cells successfully go away from repellents and others unfortunately go back to repellents but these cells go away from repellents again soon. As a whole, the

cellular population succeeds in escaping.

The molecular mechanism of escape behavior of *C. aggregans* has still been unclear. However, if chemotactic system works in the escape behavior, *C. aggregans* would possess chemoreceptor and signal transduction pathway that control motility mechanisms. In chapter 1, cell aggregation of *C. aggregans* was promoted by partial cell degradation products containing peptides. The small peptides in the degradation products may work as the repellent in the escape behavior.

Finally, I propose ecological aspects of the findings in this chapter. *C. aggregans* cells successfully avoid lysis by predators but a part of the cells are degraded to supply nutrients into the microbial community including predators.

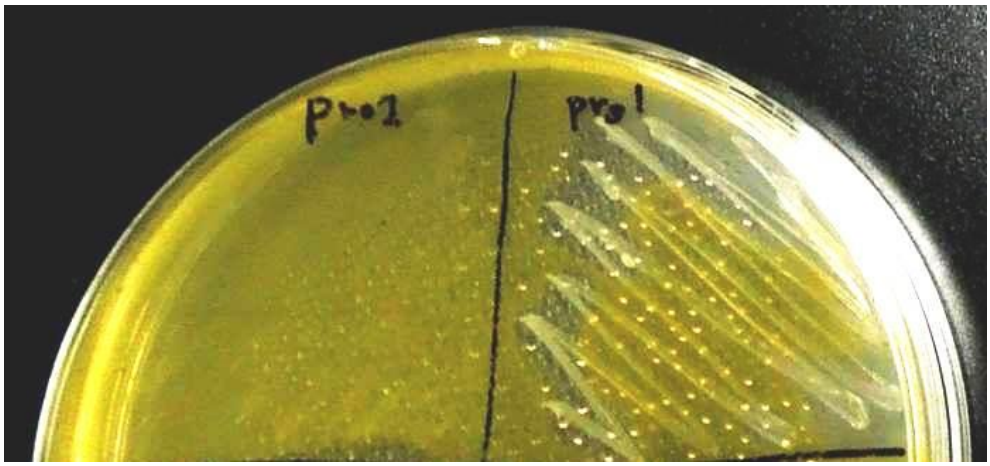
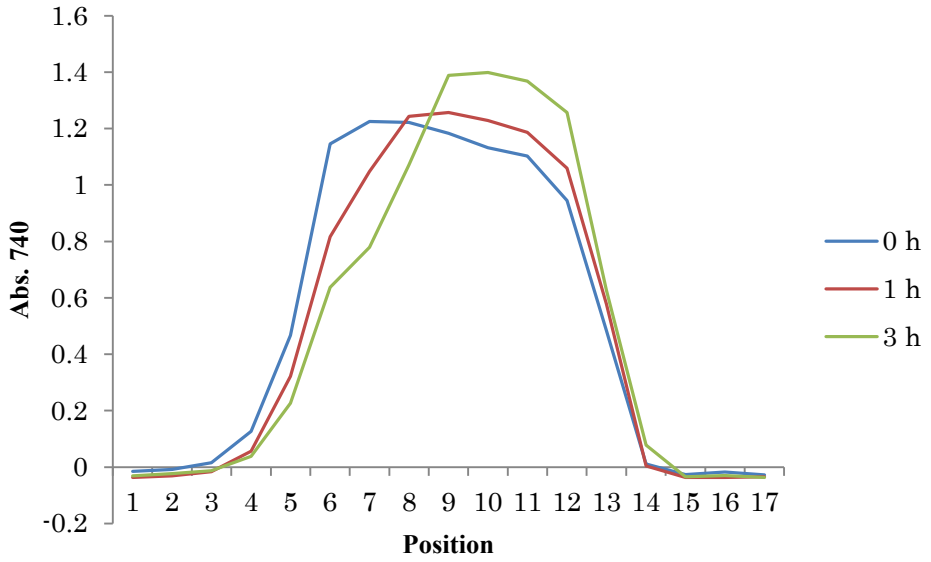


Fig. 2-1. Photoimage of the agar containing *C. aggregans* cells after cultivation of *Bacillus* sp. strain BL55a (Right) and *Aeribacillus* sp. strain NOT (Left) at 55°C.

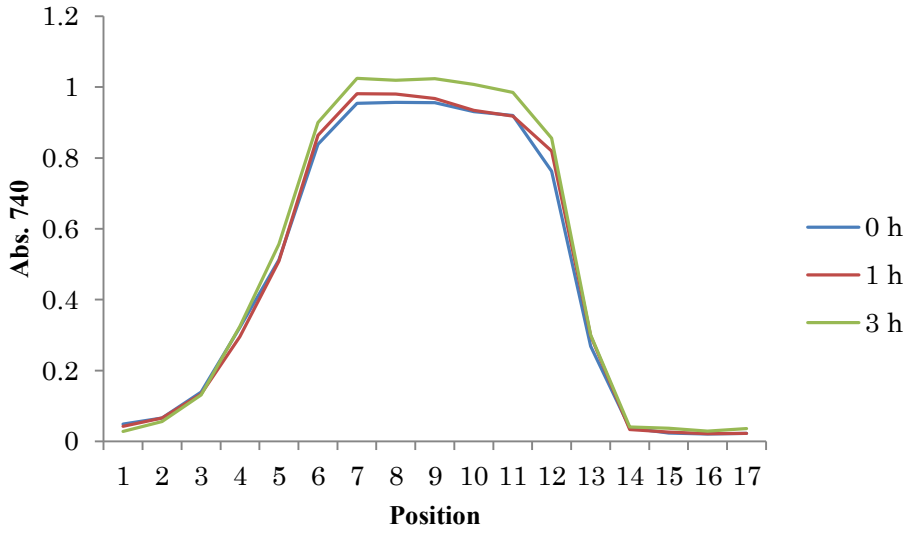
A



B

Fig. 2-2. Distribution of *C. aggregans* cell population in the cuvette containing protease in the upper layer during incubation in the light.
(A) Absorbance at 740 nm of the middle layer and the surrounding parts. The position 1 indicates the boundary between the upper layer and middle layer. Absorbance was scanned from the position 1 to the boundary of the middle layer and lower layer (position 17) at 1 mm intervals. (B) Photoimage of the cuvettes containing three agar layers, protease layer (upper), *C. aggregans* layer (middle) and fresh medium layer (lower) after 30 hours of incubation.

A



B

Fig. 2-3. Distribution of *C. aggregans* cell population in the cuvette that absence of protease in the upper layer during incubation in the light.
(A) Absorbance at 740 nm of the middle layer and the surrounding parts. The position 1 indicates the boundary between the upper layer and middle layer. Absorbance was scanned from the position 1 to the boundary of the middle layer and lower layer (position 17) at 1 mm intervals. (B) Photoimage of the cuvettes containing three agar layers, fresh medium layer (upper), *C. aggregans* layer (middle) and fresh medium layer (lower) after 30 hours of incubation.

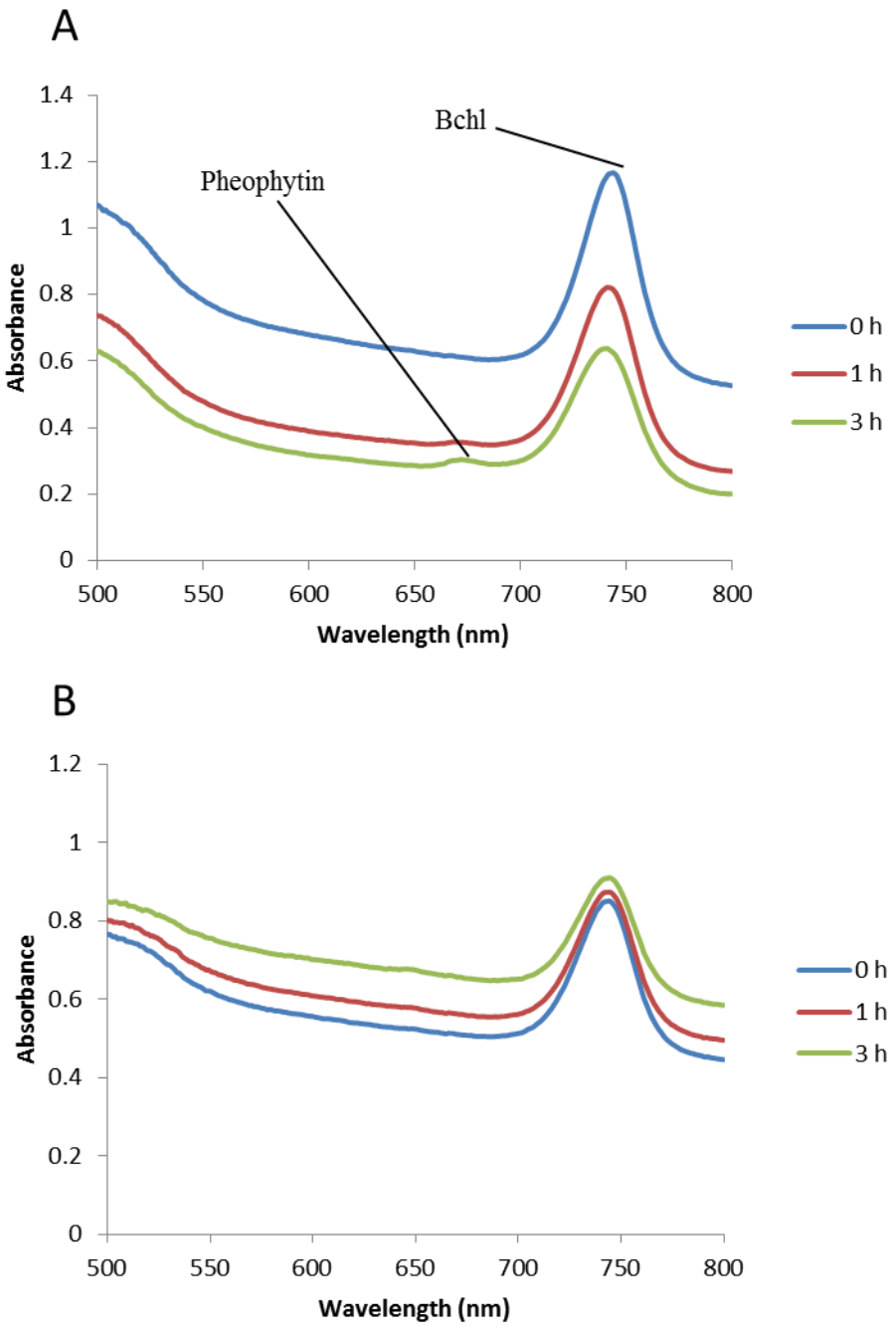
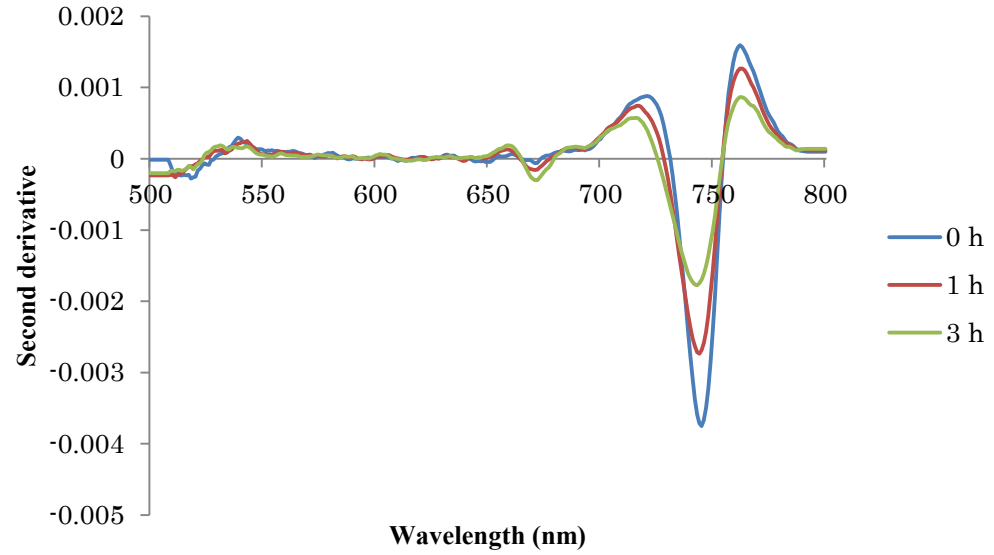


Fig. 2-4. Spectrum changes of *C. aggregans* under the cultivation

(A) Spectrum changes at the position 6 in the cuvette containing protease in the light shown in Fig. 2-2A. (B) Spectrum changes at the position 6 in the cuvette not containing protease in the light shown in Fig. 2-3A. Fig.2-4 continues next page.

C



D

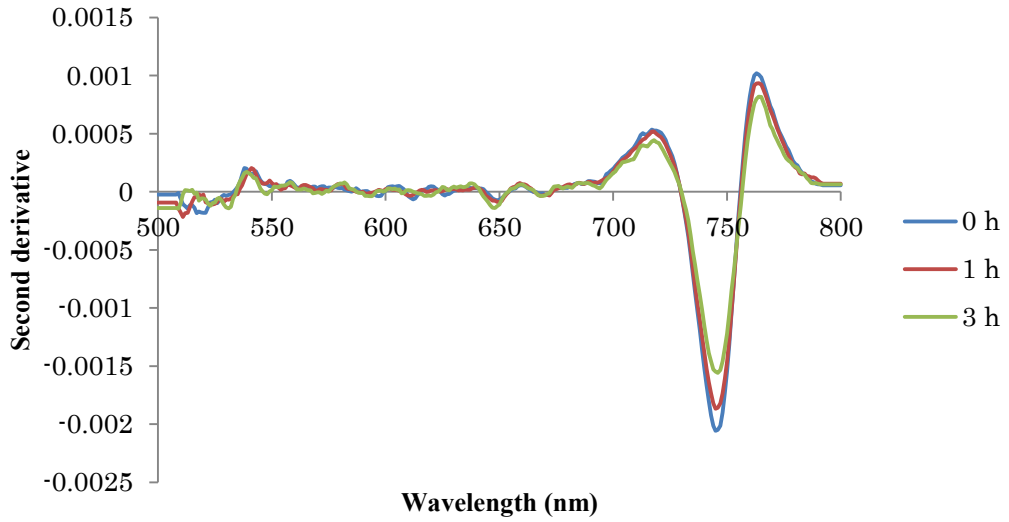


Fig. 2-4. (continued)

(C) Second derivative spectrum at the position 6 in the cuvette containing protease in the lighy condition shown in Fig. 2-2A. (D) Second derivative spectrum at the position 6 in the cuvette not containing protease in the light shown in Fig. 2-3A.

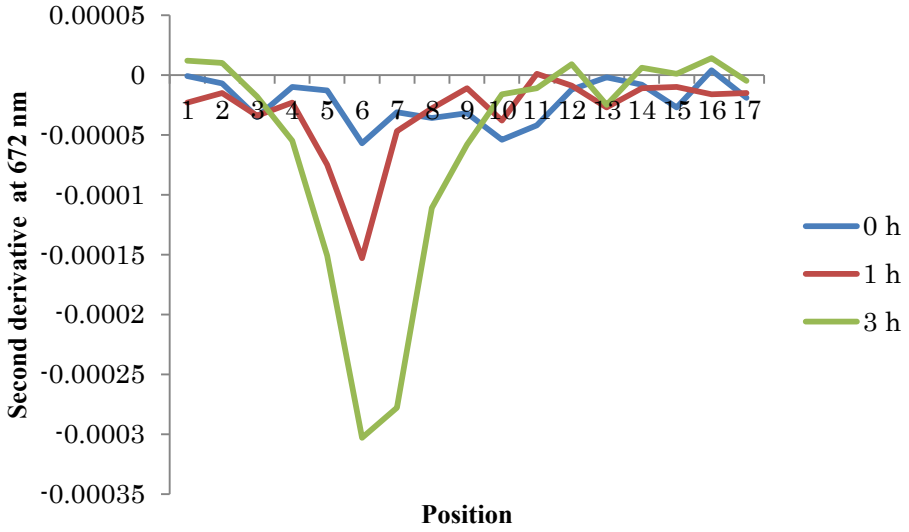


Fig. 2-5. Distribution of bacteriopheophytin in the cuvette containing protease in the light.

The second derivative of absorbance at 672 nm in the cuvette containing protease in the light shown in Fig. 2-2 was calculated.

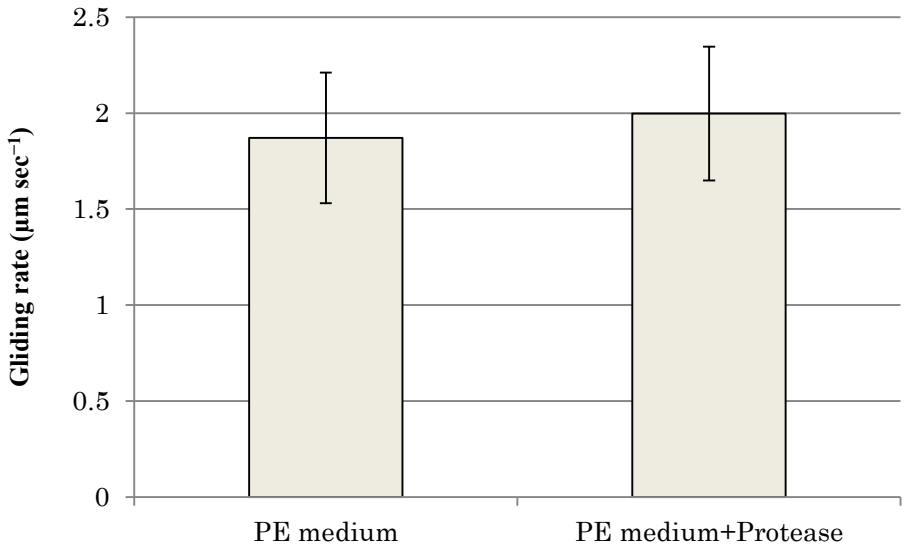


Fig. 2-6. Effect of protease on the gliding rate of *C. aggregans.*
C. aggregans were incubated with PE medium or protease containing PE medium in the glass slide at 55°C. Error bars indicate SD (n = 30).

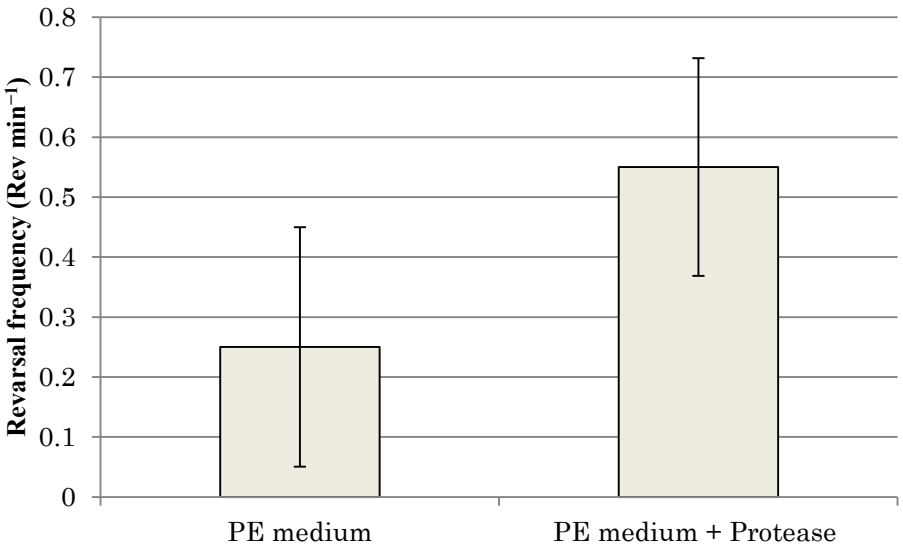


Fig. 2-7. Effect of protease on the reversal frequency of gliding movement.
C. aggregans were incubated with PE medium or protease containing PE medium in the glass slide at 55°C. The average of reversal frequencies in 10 minutes of 30 filaments was calculated. Error bars indicate SD (n = 30).

Total discussion

In this study, I found that extracellular protease secreted by other bacteria promoted cell aggregation of *C. aggregans*, and that *C. aggregans* population escaped away from protease. Extracellular proteases of bacteria play a role in obtaining nutrients through digstion of proteins. In addition to the role in own growth, my findings indicated extracellular proteases mediate interspecies interactions in bacteria.

Mechanisms of cell aggregation of *C. aggregans*

A multicellular filamentous bacterium *C. aggregans* shows rapid gliding motility (1−3 μm s^{-1}) and active cell aggregation in liquid media, while *C. aurantiacus*, a close relative of *C. aggregans*, shows slow gliding motility (0.01−0.04 μm s^{-1}) and no cell aggregation (Hanada et al., 2002). Hanada et al. proposed that gliding motility on another filament in culture solution makes them to form the cell aggregates and the gliding rate correlated with the cell aggregation rate (Hanada, 1995; Hanada et al., 2002). However, I found that the protease did not significantly affect the gliding rate but increased the cell aggregation rate (Fig. 2-6). Microscopic observation indicated that reversal frequencies of gliding direction were increased in the presence of protease (Fig. 2-7). From these findings, it can be suggested that the reversal frequencies of the filaments contributed to the formation rate of cell aggregates. In an early stage of

formation of cell aggregates in liquid media, a filament contacts on another filament by random collision and the two filaments glides on the cell surface of each other. If a filament moves without change in the direction, the two filaments will be dispersed. The high reversal frequencies could prevent the dispersion of such filaments and promote whole aggregate formation.

Protease-mediated interspecies interaction and the possibility

Exogenous protease, which had an ability to induce cell lysis changed the motility of *C. aggregans* (Fig. 1-3, Fig.2-7). How do bacteria sense extracellular proteins? As known in bacterial chemotaxis, some bacterial species possess chemoreceptor(s) at the cell surface to sense extracellular chemicals (Grebe and Stock, 1998; Koteva et al., 2010). It has not been reported that the receptors bind proteins as a signal. As shown by the results in Chapter 1, low molecular weight compounds derived from protease treatment of *C. aggregans* cells had the ability to promote the motility of *C. aggregans*. This suggests that protease indirectly work as an intercellular signal by degradation of proteins to produce peptides.

It has been known that peptides work as a cell to cell communication signal (Federle & Bassler, 2003; Lyon & Novick, 2004; Ryan & Dow, 2008). The peptides reported

previously are synthesize in a cell and are processed by a transmembrane protein to be secreted to outside of the cell (Michiels et al., 2001, Ryan & Dow, 2008). My results indicated that peptides were derived from cell surface proteins by exogenous protease. This is a novel pathway producing peptide signals through interspecies interaction.

C. aggregans and interspecies interaction in microbial mats

C. aggregans has been reported to play a primary producer in hot spring mats (Otaki et al., 2012). Organic compounds produced by *C. aggregans* are consumed by heterotrophic bacteria such as fermentative bacteria under anaerobic conditions. This study suggests that protease-producing bacteria which grow aerobically possibly utilize *C. aggregans* cells as nutrients with in the mats. Cell lysis of *C. aggregans* by extracellular protease may facilitate nutrients supply into the mat-ecosystems. Furthermore, extracellular proteases promoted the aggregate formation of *C. aggregans* possibly resulting in the densely packed structure of the mats mainly composed of *C. aggregans*. In addition, escaping behavior of *C. aggregans* from proteases as well as chemotactic behaviors responding to light and oxygen seems to be inducing continuous changes in the distribution of members within the mats.

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Conflict of Interest Statement

The author declares that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplemental data

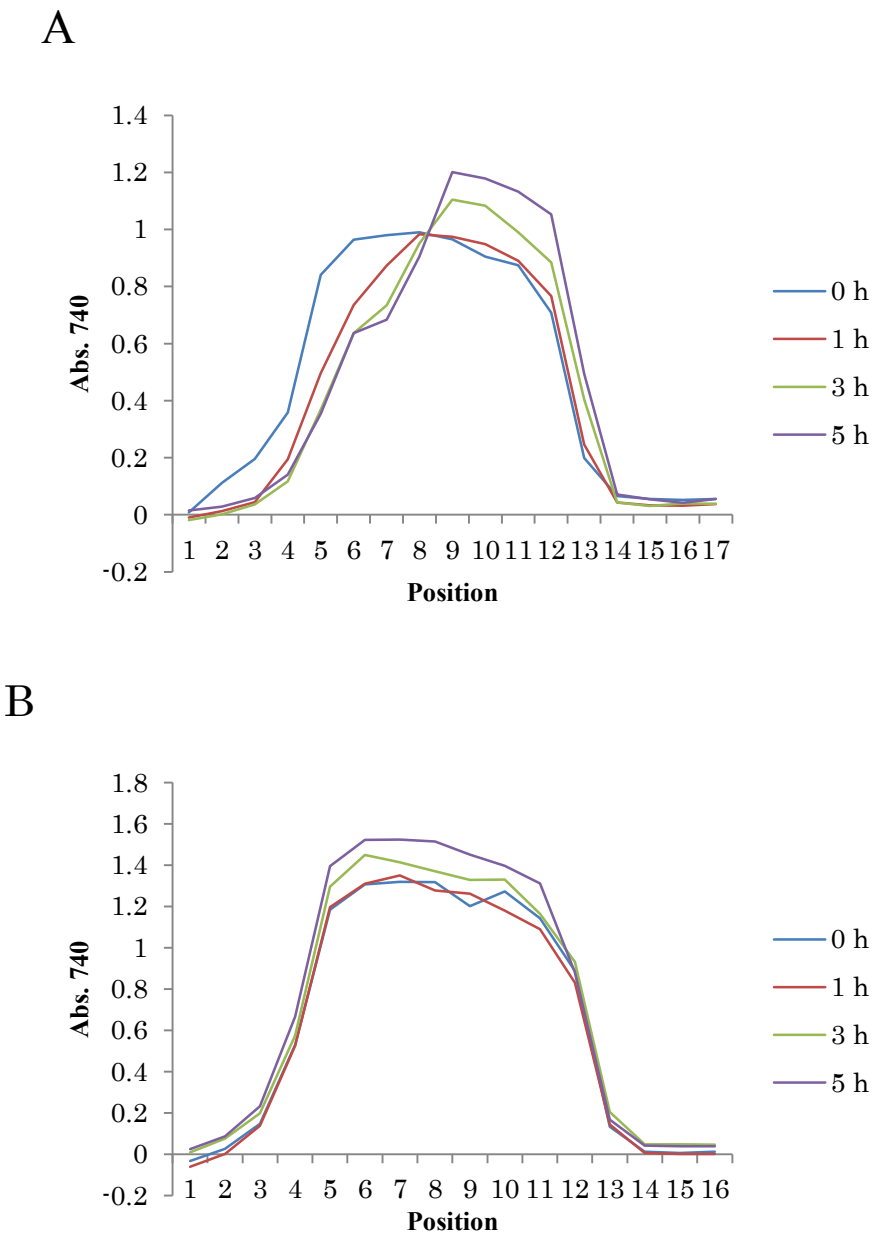

Fig. S1. Distribution of *C. aggregans* cell population in the cuvette during incubation in the light.

(A) Distribution of *C. aggregans* cell population in the cuvette containing protease in the upper layer during incubation in the light. (B) Distribution of *C. aggregans* cell population in the cuvette that absence of protease in the upper layer during incubation in the light. These experiments were conducted under the same conditions with Fig. 2-2 and Fig. 2-3.