Secreted protease mediates interspecies interaction and promotes cell aggregation of the photosynthetic bacterium *Chloroflexus aggregans*

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One-Sentence Summary: Cellular motility of a thermophilic photosynthetic bacterium was promoted by a protease secreted from other bacteria. It is a new mode of interspecies interaction in bacteria.

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**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, phenylmethylsulfonyl 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.

**Key words:** interspecies interaction; extracellular protease; cell aggregation; gliding 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 and Pier son 2006). Gliding motility is defined as the movement of a non-flagellated cell in the direction of its long axis on a surface (Spormann 1999). *Chloroflexus aggregans* MD-66T, 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-66T was influenced by light intensity, temperature and pH of the medium (Hanada, Shimada and Matsuura 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, Shimada and Matsuura 2002).
Microbial interspecies interactions cause various phenotypic outcomes including biofilm formation and cell motility (Federle and Bassler 2003; Shank and 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, Raffa and Handelsman 2006; Shank and Kolter 2009). *Chloroflexus* is widely distributed in terrestrial hot springs in the temperature range of 50–70 °C (Hanada et al., 1995b; Pierson and 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 48–65 °C (Nakagawa and Fukui 2002; Kubo et al., 2011; Everroad et al., 2012; Otaki et al., 2012). In this study, we 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′00″E, 1500 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 (30W 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 five successive plateings 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 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 (CGGTCAATTCTTGGTATT) primers (Lane et al., 1985; Lane 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 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 45 s, and extension at 72 °C for 1 min; and a final elongation at 72 °C 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 and Guijarro (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 MgCl₂, 10 mM CaCl₂ 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 × 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 A440 by 0.1 in 30 min.

**Cell aggregation test**

*C. aggregans* isolated in this study was anaerobically grown in PE medium in the light. 1 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). 6 mL of the culture supernatant was mixed with 1 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, MW > 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, MW < 10 000). (c) The effect of serine protease inhibitor was determined; phenylmethylsulfonyl 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.

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

Chloroflexus aggregans strain NBF isolated from the microbial mats at Nakabusa hot springs in Japan shared 98.7% identity with that of C. aggregans MD-66T (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-66T. As shown in Fig. 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) 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). 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); 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. 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. 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. 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. 2, bar 4). Conversely, the HMW fraction, i.e. MW > 10 000, shorten the time to about 48 min (Fig. 2, bar 5).

Effect of protease on the cell aggregation of C. aggregans

We 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), 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. 2, bars 6 and 7).

A serine protease, subtilisin prepared from B. 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. 2, bars 8 and 2); however, 100 U mL$^{-1}$ of the protease caused the cell lysis of C. aggregans (data not shown).

**DISCUSSION**

In this work, we isolated six 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 (Table 1). From the results shown in Fig. 2, we concluded that protease accelerated the cell aggregation rate of C. aggregans.

Connelly, Young and Sloma (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 and Bassler 2001; Yeo et al., 2012). In the effect of protease reported in this study, such peptides could be derived from the degradation of surface component of C. aggregans cells or components in PE medium, e.g. yeast extract. The latter may not work here, because PE medium incubated with the purified protease under appropriate conditions followed by the addition of PMSF did not promote the cell aggregation (data not shown).

As described in the results, cell lysis of C. aggregans strain NBF was observed when excess amount of the protease from B. licheniformis was applied. 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). Our findings in this study suggest a predator–prey interaction within the microbial mats between protease-releasing predators and the defensive-avoidance behavior of C. aggregans through rapid formation of cell aggregates.

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. We 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.

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Conflict of interest statement

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

REFERENCES


