Analysis of gliding motility of the filamentous bacterium

Chloroflexus aggregans

A doctoral dissertation

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Abstract

The unbranched multicellular bacterium *Chloroflexus aggregans* has the ability of rapid gliding motility. The filament moves straightforward at a constant rate on solid surface and occasionally reverses the moving direction. The gliding mechanism of this bacteria has been unclear. In this study, to understand the mechanism, I focused on multi-scale analysis of motility in *C. aggregans*, i.e., directional population movement of filaments, gliding motility of a single filament and cellular motility within a filament. This study aimed to correlate these multiple level of motility to each other.

First, I examined directional population movement of filaments of *C. aggregans* cells under oxygen gradients in the dark. Cells were suspended in soft agar medium containing a redox indicator and incubated under air or 100% O_2, respectively, in the dark. After incubation, the lower part of the test tube became to be anoxic due to oxygen consumption by *C. aggregans*, and thin band with high cell density was observed above semi-aerobic layer in both oxygen conditions. Spectroscopic analysis showed that cell density was decreased in the anoxic area after incubation. These results clearly indicate that *C. aggregans* has aerotaxis.

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In a second study, I examined the gliding motility of single filaments and cellular motility within a filament. Microscopic observation successfully detected that

glass beads attached on the cell-surface moved back and forth along the long axis of the filament. Speed of the beads movement was comparable with the gliding speed indicating that the cell-surface movement was the direct force for gliding. The bead movements on cell-surface were restricted in a single cell of a filament, and the beads moved on each cell independently. Electron microscopic observation showed two characteristic features on the cell surface; 1) flexible fibers growing from cell surfaces, and 2) many linear structures that run on cell-surface. The fiber was similar in the morphology to type 4 pilus mediating twitching motility. When fine structure on cell surface was observed by high-speed atomic force microscopy, the linear structures was observed as linear convex structures. The results of this study propose a possible model of gliding machinery that cell-surface movement mediated by pilus retraction is the direct force of gliding.

However, It is unclear how the moving direction of the filament is determined by the cellular movements that direct independently within a filament. I assumed a discrete-time stochastic models based on a postulate that the moving direction of the filament is determined only when the filament pauses by sum of the directions of the cellular movements. In this model, separate directions of the cellular movements did not have a tug of war, i.e., the filament keeps moving in a unidirectional manner as long as

at least one cell moves to the direction. Monte Carlo simulation of this model showed that reversal frequency of longer filaments was relatively fixed to be low, but the frequency of shorter filaments varied widely. This simulation result appropriately explained experimental observations.

Finally, I proposed the models that explain multi-scale motility of the multicellular filament in *C. aggregans* comprehensively.

Contents

Chapter 3

Determinants of moving direction in a multicellular filamentous bacterium *Chloroflexus aggregans*

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General Introduction

Gliding motility of a multicellular filamentous bacterium, *Chloroflexus aggregans*

Chloroflexus aggregans is a thermophile and can grow by aerobic respiration in the dark and anoxygenic photosynthesis in the light (Hanada 2014). *Chloroflexus* species are widely found in microbial mats in alkaline hot springs where bacterial cells are densely packed (Hanada et al., 1995b). Cells of *Chloroflexus* form un-branched multicellular filaments, and possess gliding motility, defined as non-flagellated movement on solid surface (Hanada 2014; Spormann 1999). This motility and filamentous morphology is widely found in the phylum *Chloroflexi*, and *C. aggregans* is the most rapid glider in this phylum (Hanada 2014).

The cells of *C. aggregans* (1.0-1.5 μm wide; 2-6 μm long) form filaments of indefinite length ranging up to 400 μm, and move straightforward along long the axis of the filament at constant speeds of 1 - 3 μm/sec at 55˚C (Hanada et al., 1995a). The filamentous cells occasionally change the moving direction. It is also known as a remarkable feature of *C. aggregans* that this bacterium can rapidly form a bacterial mat-like dense cell aggregate in a liquid medium, mediated by rapid gliding motility of this bacterium (Hanada et al. 2002). Morohoshi et al. (2015a) found that the cell aggregation was promoted by protease secreted from other bacteria isolated from the

bacterial mat where *C. aggregans* were dominated. Morohoshi (2015b) also reported an escape behavior of *C. aggregans* from protease. These suggest that filaments of *C. aggregans* glide within the microbial mats that composed by *C. aggregans* and other bacteria, and the motility enhances survivability by tactic behavior and formation of cell aggregate.

Mechanical diversity of gliding motility

Gliding motility has been found in various bacteria, and the diverse mechanisms have been gradually revealed in the recent studies of unicellular gliding bacteria such as *Mycoplasma*, *Flavobacterium,* and *Myxococcus* (Miyata 2010; Nan et al. 2014; Shrivastava et al. 2015). *Mycoplasma mobile* "walks" over surface with consecutive conformational changes of cell surface adhesins like "animal feet". Otherwise, it was suggested that the gliding of *Flabobacterium jhonsoniae* was mediated by adhesins that migrate helically around cell surface (Nakane et al., 2013). *Myxococcus xanthus* has two motility systems that are called Social (S) motility and Adventurous (A) motility (Shrivastava et al. 2015). S-motility, that appears when the cells are in high cell density, is mediated by extension and retraction of pili growing from the cells, as does bacterial "twitching motility" that is movement in a somewhat

jerky fashion on solid surface. Two models have been proposed as the mechanism of A-motility that appears when the cells are in low cell density (Shrivastava et al. 2015). One model postulates that cell-surface adhesins is propelled by the motors on the cell envelope, resulting in cell movement. In the second model, deformation of the peptidoglycan layer and outer membrane mediate bacterial gliding.

Although the gliding motility has been also found in multicellular bacteria, only a few reports have dealt with gliding of multicellular filamentous bacteria. No gliding machinery of filamentous bacteria has been identified yet, and the gliding mechanisms are still mysterious. Microscopic studies on filamentous cyanobacteria proposed two gliding models; (a) pushing substratum by spouting slime from cell poles (Hoiczyk and Baumeister 1998) and (b) rhythmical undulations of cell surface along the filament push exuded slime (Halfen and Castenholz 1971). Ridgway and Lewin (1988) detected multiple cell surface movements in *Flexibacter polymorphus* in the phylum *Bacteroidetes* (Ridgway and Lewin 1988). The movement traced a helical path along the filament surface. In filamentous gliding bacteria belonging to the phylum *Chloroflexi*, the cell surface morphology has not been studied, and the gliding machinery has been completely unclear.

Chemotaxis in gliding bacteria

Many bacteria use motility as an adaptation to their environments. Bacterial chemotaxis is a tactic behavior of motility in which cells move in the direction of higher concentrations of an attractant and away from repellents, in order to move towards a favored environment and enhance their survivability (Bray, 2001). The mechanism of chemotaxis in gliding bacteria was first reported in 1998 in the study of *Myxococcus xantus*, an unicellular bacterium belonging to the phylum *Proteobacteria* (Daniel et al., 1998). The chemotactic behavior was controlled by frequency of change in the moving direction, i.e., how often bacterial cells change the direction. When the cells are exposed to concentrated chemo-attractant, reversal frequency firstly decreases, gently increases, and finally, recovers to the frequency that is same as without the attractant. The second and third step are called "adaptation". Increment of reversal frequency and adaptation are unifying principles both among swimming and gliding bacteria.

In this study, I focused on multi-scale analysis of motility in *C. aggregans*, i.e., directional population movement of filaments, gliding motility of a single filament and cellular motility within a filament. As a directional population movement, I found that *C.*

aggregans cells migrated toward the environment of optimum oxygen concentration (Chapter 1). I successfully detected cell-surface movements of *C. aggregans* using microscopic observations and characterized the cell surface morphology by electron microscopy and atomic force microscopy (Chapter 2). I analyzed the cell-surface movements of *C. aggregans* and clarified the effect of filament lengths on gliding movements (Chapter 3). Finally, I propose a relevant mechanism explaining how the multicellular filamentous bacteria can move straightforward at a constant rate and occasionally change the moving direction.

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Chapter 1

The relationship between spatial cell distribution and aerotactic behavior of a gliding anoxygenic photosynthetic bacterium *Chloroflexus aggregans*

Abstract

The anoxygenic photosynthetic bacterium *Chloroflexus aggregans* has the ability of aerobic respiration and gliding motility. *C. aggregans* is often found in bacterial mats developed in hot spring waters. *C. aggregans* is heterogeneously distributed in the bacterial mats where oxygen gradient is likely formed from the surface to the bottom. I examined the change in distribution of *C. aggregans* cells in test tubes to clarify the aero-tactic behavior. *C. aggregans* cells were suspended with soft agar medium containing a redox indicator, resazurin in test tubes and incubated under air in the dark. After 12 h of incubation, a thin band with high cell density in the agar medium was observed. The position where the band was observed was the boundary between aerobic and anaerobic area. When incubated under 100% O_2, formation of the thin band was similarly observed at the aerobic-anaerobic boundary which appeared at a deeper position. Spectroscopic analysis of the agar medium at each depth confirmed that the cell density was decreased in the anaerobic area and was increased at the semi-aerobic position. These results clearly indicated that *C. aggregans* migrated to semi-aerobic environments through the aero-tactic movement.

Introduction

A thermophilic filamentous bacterium *Chloroflexus aggregans* is an anoxygenic photosynthetic bacterium which is widely found in terrestrial hot springs (Hanada et al., 1995). This bacterium also has the ability of heterotrophic aerobic respiration. Cells of *C. aggregans* form unbranched multicellular filaments (30-400μm in length), and possess gliding motility, i.e., non-flagellated movement on solid surface (Hanada, 2014; Spormann, 1999). *C. aggregans* moves along the long axis of their filament at speeds of 1 - 3 μm/sec at 55°C (Hanada et al., 1995; Morohoshi et al., 2015). The filament glides on other filaments, and forms a dense cell-aggregate in liquid medium (Hanada et al., 2002).

Chloroflexus spp. have been found in microbial mats, where bacterial cells are densely packed. At Nakabusa hot springs in Japan, *C. aggregans*-dominated mats are developed in hot spring water at 50 - 70°C. Kubo et al. (2011) reported heterogeneous vertical distribution of bacteria within the mats; aerobic sulfide-oxidizing bacteria were near the mat surface, while anaerobic sulfate-reducing bacteria were in the deeper layer. These observations strongly suggested that the mats were spatially separated into aerobic top layer and anaerobic area. *C. aggregans* widely distributed within the mats, but its population both in the aerobic surface and anaerobic bottom were limited (Kubo

et al., 2011). *C. aggregans* may preferably migrate to appropriate part via its gliding motility depending on environmental factors such as dissolved oxygen concentration. However, the relationship between distribution of *C. aggregans* and the oxic/anoxic environments has been unclear, so far.

Aerotaxis is a behavior of cell motility along gradients of oxygen concentration and aerotactic bacterial cells move toward optimal oxygen concentration (Bray, 2001). The optimal oxygen concentration correlates with the metabolic requirements for oxygen (Taylor et al., 1999). Because concentration of oxygen is a critically important factor for cell metabolism and growth, aerotaxis is advantageous for cell survival (Mazzag et al., 2003). Aerotaxis has been reported in many swimming bacteria such as *Escherichia coli*, but it has not been studied in gliding bacteria. I hypothesized that *C. aggregans* glides within a microbial mat, and the motility helps to migrate to the optimal oxygen condition.

In this chapter, I examined the change in distribution of *C. aggregans* cells under oxygen gradients in the dark, and clarified the aerotactic behavior through the gliding motility *in C. aggregans*.

Materials and methods

Bacterial strain and growth conditions

     Chlorofexus aggregans strain NBF used for this investigation was previously isolated from Nakabusa Hot Springs in Japan (Morohoshi et al., 2015). The cells were anaerobically grown in PE medium (Hanada et al., 1995) at 55°C under incandescent light. PE medium consisted of the following components (per liter); 0.5 g sodium glutamate, 0.5 g sodium succinate, 0.5 g sodium acetate, 0.5 g yeast extract (Difco), 0.5 g casamino acids (Difco), 0.5 g sodium thiosulfate, 0.38 g of KH_2PO_4, 0.39 g of K_2HPO_4, 0.5 g of $(NH_4)_2SO_4$, 1 ml of vitamin mixture, and 5 ml of basal salt solution. The pH of the medium was adjusted to 8.0.

Incubation of C. aggregans *cells in soft agar medium in a glass test tube*

     1 ml of cell suspension of *C. aggregans* photo-heterotrophically grown in PE medium to exponential-growth phase were collected by centrifugation at $7,000 \times g$ for 10 min. The cells were suspended into 10 ml of PE medium containing 0.7% (w/v) agar (Wako) and 0.04% resazurin as an oxidation-reduction indicator. The soft agar medium containing *C. aggregans* cells was funneled into 32 ml-glass test tube (the diameter of 1.8cm) and solidified in air. Depth of the medium in the test tube was 5 cm and height

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of air phase in the tube was about 12 cm. The air phase was replaced with 100% O_2 or 100% N_2 gas if necessary. The tubes were incubated at 55 ˚C in the dark.

Incubation of C. aggregans *cells in soft agar medium in a cuvette*

   C. aggregans was cultivated and the cells were collected as described above. The cells were suspended in 1ml of PE medium containing 0.05% agar and 0.04% resazurin. 140 µl of the soft agar medium containing *C. aggregans* cells was solidified in a quartz cuvette sealed with silicone rubber stopper (the optical path length of 1 mm; the width of 10 mm; the height of 58 mm, GL Sciences, Japan, Tokyo). The air phase was replaced with 100% O_2 gas. The cuvette was incubated at 55 ˚C in the dark. Absorption spectra of the medium at every 1mm-depth in the cuvette were periodically recorded during incubation with a spectrophotometer (Shimadzu UV1800, Japan, Kyoto).

Results

Effect of oxygen on migration of C. aggregans *in soft agar medium*

  C. aggregans cells were suspended with 0.7% soft agar medium and incubated under air for 12 hours in the dark to observe the migration in agar in a glass test tube. Figure. 1-1 shows sequential images of the test tubes. At the beginning, the cell suspension containing resazurin showed the pink color indicating aerobic conditions from the bottom to the agar surface. After 5 hours of incubation, the pink color at the lower part disappeared as shown in Figure. 1-1. This indicated that the lower part became anoxic. This can be explained by the oxygen consumption by *C. aggregans* which lives by aerobic respiration under dark conditions. Position of the boundary between pink layer and greenish layer was approximately 0.8 cm depth of the agar medium. The boundary did not change during incubation for 12 hours. After 12 hours of incubation, a thin band with high cell density was observed at the approximate position of the boundary between pink and greenish layers.

  The same experiment was conducted after the gas phase in the test tube was replaced with O_2 gas. As shown in Figure. 1-2a, the boundary between pink and greenish layers appeared at deeper position, i.e., 2.1 cm depth from the agar surface. *C. aggregans* cells accumulated to the boundary as observed in Figure. 1-1. When the gas

phase was replaced with N$_2$ gas, pink color of resazurin disappeared within 5 h of incubation and no definite accumulation was observed (Fig. 1-2b).

Chasing the migration by spectroscopic analysis

Spectroscopic analysis of the agar medium suspended with *C. aggregans* cells and resazurin was able to show quantitative changes in cell density and oxic/anoxic conditions. Figure. 3 shows absorption spectra of the pink layer (blue line) and the greenish layer (red line) of cell suspension after 30 min of incubation. The absorption peaks at 571 nm and 740 nm indicated oxidative state of resazurin and bacteriochlorophyll c of *C. aggregans* cells, respectively. The figure shows that absorbance at 660 nm was not affected by rezazurin and bacteriochlorophyll that could change during incubation in the dark. Thus, absorbance at 660 nm was chosen as indicator of cell density. Although the absorbance at 571 nm should be increased as bacterial density, 3 peaks in quadratic differential spectrum (negative peak at 571 nm; positive peak at 589 nm and 562 nm) indicating the redox states of resazurin should be independent of the cell density.

In order to quantitatively determine absorbance, agar medium containing *C. aggregans* cells was solidified in a cuvette of which the gas phase was replaced with O$_2$.

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Quadratic differential absorbance at 589 nm for resazurin and absorbance at 660 nm as an indicator of cell density of every 1 mm of depth from the agar surface were periodically determined during incubation (Fig. 1-4). Quadratic differential absorbance at 589 nm indicated that the agar medium in the cuvette was divided into oxic layer at 1-6 mm depth, semi-oxic layer at 7 mm depth and anoxic layer at 8-10 mm depth (Fig. 1-4a). Further incubation shifted the semi-oxic layer upward, and slightly decreased the absorbance at the top layer. Increase in absorbance at 660 nm of the layer at 1-5 mm depth and decrease in the absorbance of the layer at 8-11 mm depth were observed after 3.7 h of incubation comparing with the values at 0.5 h of incubation (Fig. 1-4b). After 6.7 h, further increase in the absorbance was observed at 2-4 mm depth and the absorbance at the other parts was decreased. Largest increase was detected at 4 mm depth which was the upper next to the semi-oxic layer.

Discussion

This study found that the cell density of *C. aggregans* increased at the approximate position of the oxic-anoxic boundary. The accumulation of cells should attribute to the cell migration toward the micro-aerobic condition, because photometrical analysis showed the cell density in adjacent areas to the micro-oxic area, i.e., oxic area and anoxic area, decreased. This result clearly indicates aero-tactic movements of *C. aggregans*. This is the first report that detects aero-taxis of gliding bacteria in pure culture.

A possible mechanism of tactic behavior to chemo-attractants in a gliding bacterium was proposed for *Mxococcus xantus* belonging to the phylum *Proteobacteria* (Daniel et al., 1998) as follows; when the cells sense chemo-attractants, the cells moved straightforward for a while; when the cells reached to the optimal concentration of chemo-attractants, the cells increased reversal frequency to stop; when the cells moved away from the optimal concentration of chemo-attractants, the cells changed the moving direction to move back to the optimal concentration. This processes are likely applicable to aero-tactic behavior of *C. aggregans* shown in Figure. 1-4.

Aerotactic migration has been reported for many swimming bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, and *Azospirillum brasilense* (Shioi et al.,

1988; Barak et al., 1982). The physicochemical parameters of the area which the aerotactic bacteria migrate to may be correlated with metabolic requirement of oxygen, because oxygen is critical to cell metabolism and growth (Taylor et al., 1999). *C. aggregans* migrated to the area near the micro-oxic layer. *C. aggregans* grows by aerobic respiration in the dark, whereas it does by anaerobic-photosynthetic in the light (Hanada, 1995). In *C. aggregans*, oxygen is essential for aerobic respiration in the dark but should be rather toxic for anaerobic photosynthesis in the light. Thus, the migration should be strictly controlled depending on the optimum oxygen concentrations for metabolic requirement.

Aerotaxis plays an important role in bacterial distribution within microbial mats (Barry et al., 1999). Doemel et al. reported the diurnal distribution change of *Chloroflexi* within a mat where *Chloroflexi* co-exist with cyanobacteria (Doemel et al. 1977). They suggested that the distribution change was achieved by aerotaxis of *Chloroflexus* sp. responding to dynamic change of oxygen concentration through oxygenic photosynthesis by cyanobacteria, but no one has shown the aero-tactic behavior of *Chloroflexus* in pure culture. Kubo et al. (2011) reported the distribution of *C. aggregans* within a bacterial mat where no cyanobacteria co-existed. Aero-tactic migration found in this study likely explains the distribution, i.e., the population of *C.*

aggregans in the anaerobic bottom was limited, and that in the middle position was

higher than that at the aerobic surface, although other factors such as light should be

considered simultaneously. Aerotaxis of *C. aggregans* found in this study may work to

obtain organic compounds which may be supplied by sulfur-oxidizing bacteria at the

aerobic surface even in the dark resulting in increase in survivability of *C. aggregans*.

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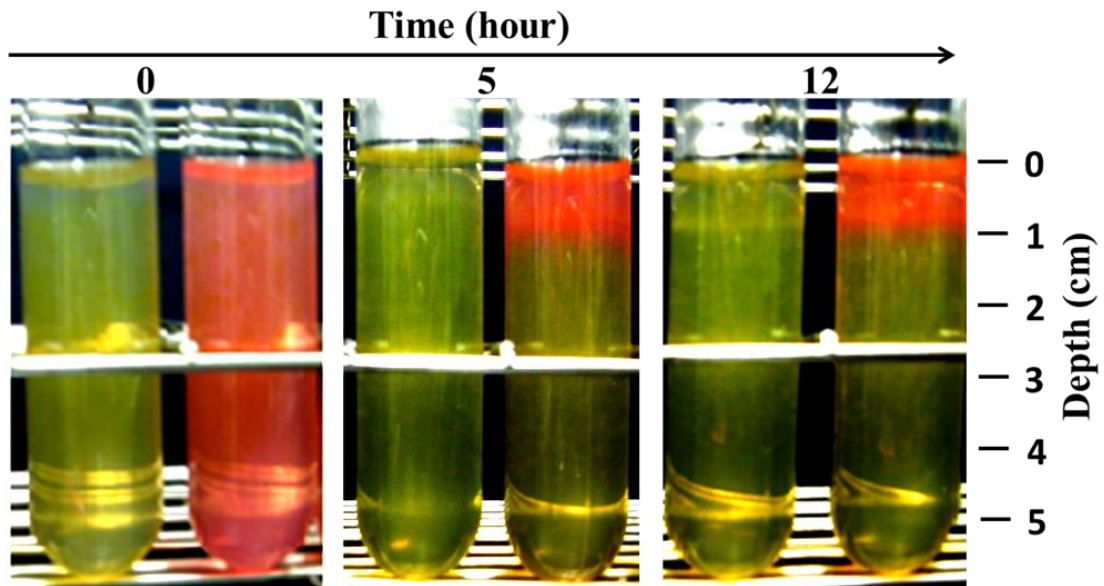
Figures and legends

Fig.1-1 Sequential photographs of agar medium containing *C. aggregans* cells

in glass test tubes. The gas phases in all test tubes were air. Test tubes in right at each

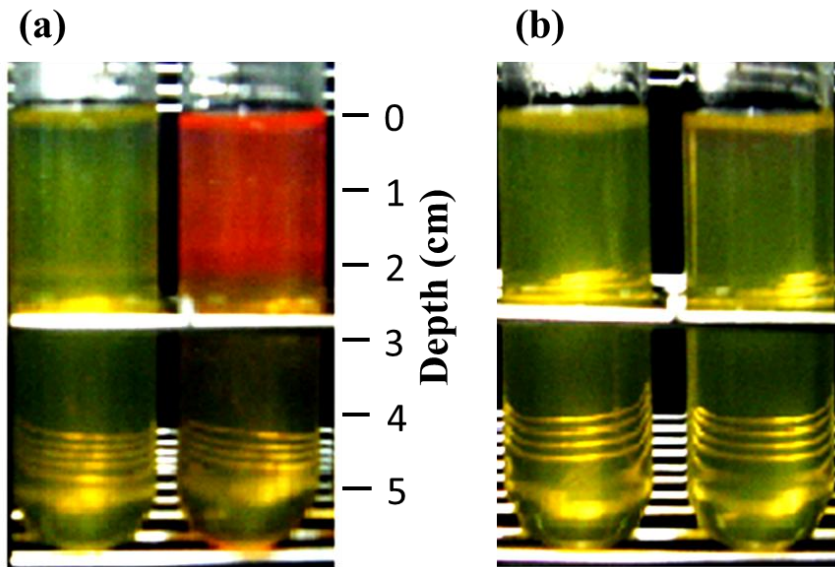time point contained resazurin.

Fig.1-2 Photographs of agar medium containing *C. aggregans* cells in glass test tubes after 12 h incubation. (a) The gas phases were filled with 100% O_2 gas. (b) the gas phase were filled with 100% N_2 gas. Test tubes in right at each figure contained resazurin.
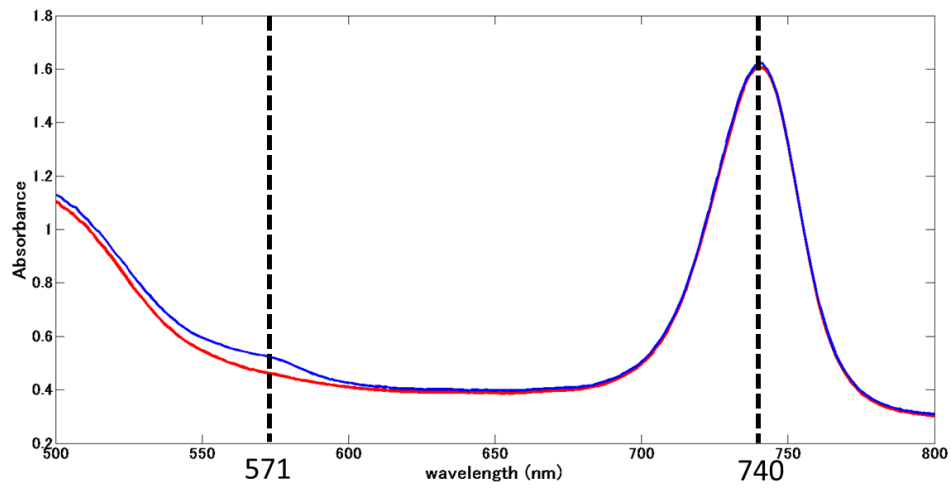
Fig.1-3 The absorption spectra of agar medium suspended with *C. aggregans* cells and resazurin. Blue line and red line show the spectrum at oxic layer and anoxic layer after 30 min incubation, respectively.
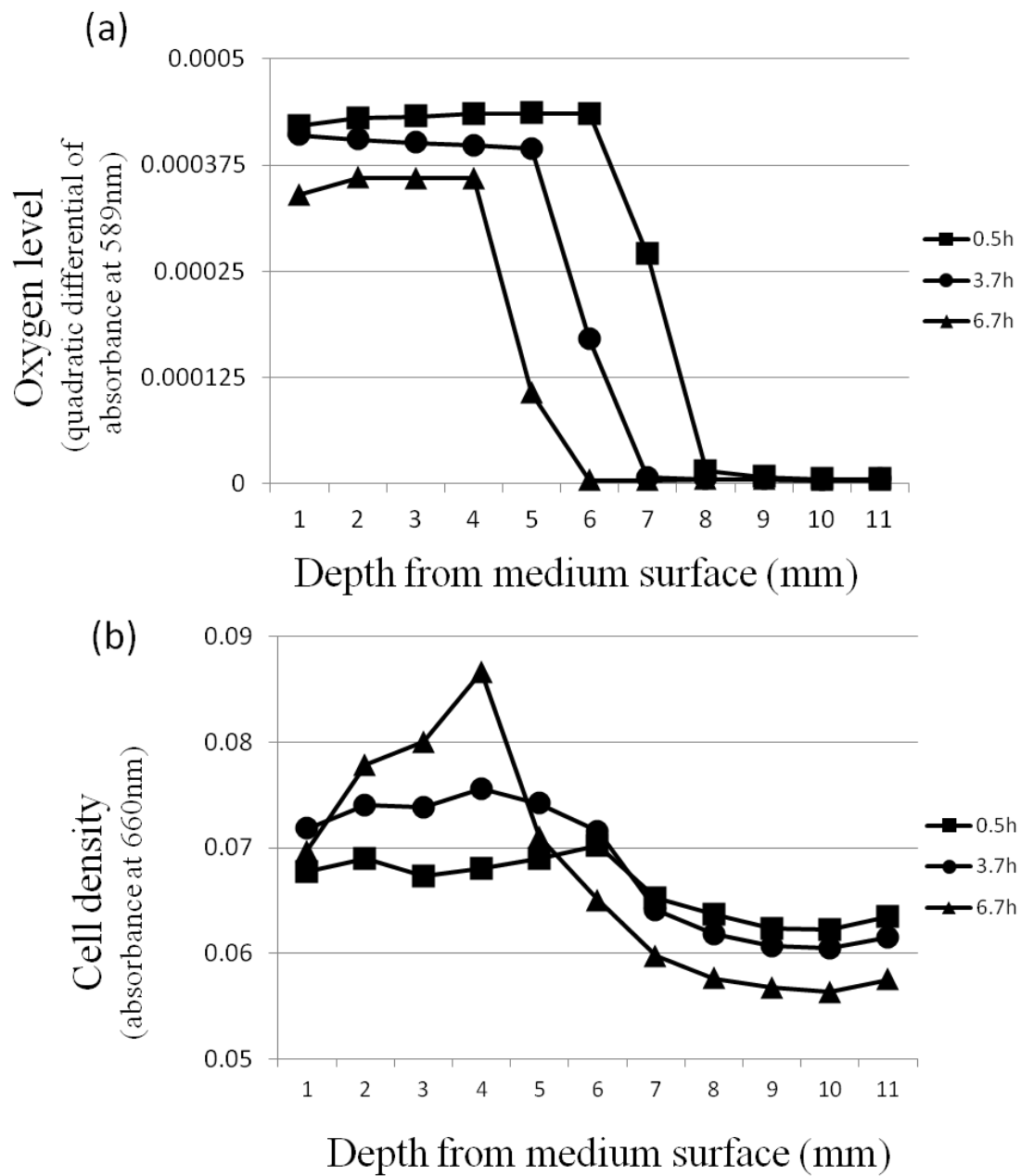
Fig. 1-4 Quadratic differential absorbance at 589 nm (a) of absorption spectrum

and absorbance at 660 nm (b) of agar medium containing *C. aggregans* cells and

resazurin in cuvettes. The absorbances were determined at each depth after 0.5h, 3.7h

and 6.7h of incubation.

Chapter 2

Cell-surface movements and the related cellular structures of

a gliding filamentous bacterium *Chloroflexus aggregans*

Abstract

Chloroflexus aggregans is an unbranched multicellular filamentous bacterium with the ability of gliding motility. The filament moves straightforward at a constant rate, approximately 3 μm/sec on solid surface and occasionally reverses the moving direction. In this study, microscopic observation successfully detected movements of glass beads attached on the cell-surface along the long axis of a filament. The beads oscillated back and forth within a distance of 2.5-6.5 μm of the distance. Speed of the movement of beads was determined to be 2.70±0.33 μm/sec, which was comparable with the gliding speed of filaments. It indicated that the cell-surface movement was the direct force for gliding. Electron microscopic observation showed two characteristic morphological features of cell surface; 1) flexible fibers growing from cell surfaces and 2) many linear structures that run on cell-surface. The fibers were several μm in length and approximately 5 nm in diameter. It was quite similar to type 4 pili, which mediate twitching motility. Linear structures that run on cell-surface was also observed by high-speed atomic force microscopy. The observation indicated that the linear structures was attributed to linear furrows or/and convex structures on cell surface along the long axis. This study proposed that cell-surface movement mediated by pilus retraction was a direct force of gliding as a possible model of gliding machinery of *C. aggregans*.

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Introduction

Chloroflexus aggregans is a filamentous anoxygenic photosynthetic bacterium in the phylum *Chloroflexi*. The cells (1.0-1.5 μm wide; 2-6 μm long) form unbranched multicellular filaments of indefinite length, that the length excess of 400 μm in some filaments. The filamentous morphology is widely found in the phylum *Chloroflexi* (Hanada 2014). Gliding motility, non-flagellated movement on solid surface, has been reported for phototrophic groups (the families *Chloroflexaceae*, *Roseiflexaceae* and *Oscillochloridaceae*) and some species of non-phototrophic bacteria in this phylum (Hanada, 2014). *C. aggregans* isolated from terrestrial hot springs moved straightforward along the long axis of a filament at speeds of 1 - 3 μm/sec at 55˚C (Hanada et al., 1995), which is more than 10 times faster than other bacteria belonging to the phylum *Chloroflexi*.

Although gliding motility of unicellular bacteria such as *Flavobacterium*, *Myxococcus* and *Mycoplasma* has been well studied (Shrivastava et al., 2015; Nan et al., 2014; Miyata, 2010), only few reports dealt with gliding of multicellular filamentous bacteria. No gliding machinery of filamentous bacteria has been identified yet and the gliding mechanisms are still mysterious. Microscopic studies on filamentous cyanobacteria proposed two gliding models; (a) pushing substratum by spouting slime

from cell poles (Hoiczyk and Baumeister, 1998) and (b) rhythmical undulations of cell surface along the filament push exuded slime (Halfen and Castenholz, 1971). Ridgway and Lewin (1988) detected multiple cell surface movements in *Flexibacter polymorphus* in the phylum *Bacteroidetes*. The movement traced a helical path along the filament surface. Although cell surface morphology is critical for gliding ability, no experimental results have been reported in filamentous bacteria in the deeply branching phylum, *Chloroflexi*. Genome analysis of *C. aggregans* did not suggest the existence of genes related to gliding machineries which have been reported for other known gliding bacteria (Klatt et al., 2007).

In this study, I detected the cell-surface movements of *C. aggregans* to characterize the speed and direction which were closely related to the gliding motility and visualized the cell surface morphology using electron microscope and atomic force microscope.

Materials and methods

Organism and growth conditions

Chloroflexus aggregans strain NBF used for this investigation was previously isolated from Nakabusa Hot Springs in Japan (Morohoshi et al., 2015). The cells were anaerobically grown in PE medium (Hanada et al., 1995) at 55°C under incandescent light.

Analysis of cell-surface movements

Micro-glass beads (1.32 μm of median size, Nihon Horo Yuyaku, Tokyo, Japan) were suspended in 0.05% poly-L-lysine solution, and incubated at room temperature for 1 hour. The beads were washed three times with distilled water and suspended in PE medium. Culture solution of *C. aggregans* was mixed with the beads solution and centrifuged (4,000 × g for 2 min). The precipitate including the bacterial cells and beads was gently re-suspended in fresh PE medium. The suspension was poured into a 'tunnel' (16-mm interior width, 18-mm length, and 1-mm wall thickness) constructed by taping a coverslip to a glass slide using double-sided tape, following the method described by Uenoyama et al. (2004). The movements of glass beads attached on bacterial cells were observed under a phase-contrast microscope (AXIO Imager A2,

Carl Zeiss, Oberkochen, Germany) equipped with a heating stage at 55°C (Panel Glass

Heater, BLAST, Kanagawa, Japan). Micrographs were recorded with a DP73 camera

(Olympus, Tokyo, Japan) and analyzed using CellSens standard software (Olympus)

and Image J 1.46r (National Institutes of Health, Maryland, USA).

Scanning electron microscopic analysis of cells coated with osmium

C. aggregans cells were collected in exponential growth phase by

centrifugation at 4,000 × g for 2 min. The collected cells were fixed with 2%

paraformaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4°C

overnight. The samples were additionally fixed with 1% tannic acid in 0.1 M cacodylate

buffer pH 7.4 at 4 °C for 2 h. After the fixation, the samples were rinsed 4 times with

0.1 M cacodylate buffer for 30 min, followed by post fixation with 2% osmium

tetroxide in 0.1 M cacodylate buffer at 4 °C for 3 h. The samples were dehydrated

through a series of graded ethanol (50%, 70%, 90%, 100%). The schedule was as

follows: 50% and 70% for 30 min each at 4°C, 90% for 30 min at room temperature,

and 4 changes of 100% for 30 min each at room temperature. Then, the samples were

continuously dehydrated with 100% ethanol at room temperature overnight. The

dehydrated samples were substituted into tert-butyl alcohol at room temperature. The

schedule was as follows: 50:50 mixture of ethanol and tert-butyl alcohol for 1 h, 3

changes of 100 % tert-butyl alcohol for 1 h each followed by being frozen at 4°C. The

frozen samples were vacuumed dried. After drying, the samples were coated with a thin

layer (30 nm) of osmium by using an osmium plasma coater (NL-OPC80NS, Nippon

Laser & Electronics Laboratory, Japan). The samples were observed by a scanning

electron microscope (JSM-6340F) at an acceleration voltage of 5.0 kV.

Transmission electron microscopic analysis of negatively stained cells

C. aggregans cells were collected in exponential growth phase by

centrifugation at $4,000 \times g$ for 2 min and re-suspended in a fresh PE medium. 5μl of the

cell suspension was dropped on a laboratory film, and a Ni grid supported by carbon

film was floated on the drop. After the grid on the drop of cell suspension was incubated

in a moist chamber at 55°C in the light for 10 min, the cell suspension was washed out

with enough volume of 5 % ammonium molybdate, and the grid was floated to 5 μl of

5% ammonium molybdate that preliminarily dropped on a laboratory film for 1 sec.

This step repeated twice. Residual ammonium molybdate was gently removed using

filter paper, and the grid was dried at room temperature. Micrographs were taken with a

JEOL model JEM-1400 electron microscope operating at 80 kV.

High-speed atomic force microscopy

1) Sample preparation

C. *aggregans* cells were collected in exponential growth phase by centrifugation at 4,000 × g for 2 min and re-suspended in the same volume of the fresh PE medium, then were concentrated 30 times by centrifugation. A mica disc (1.5 mm in diameter; Furuuchi Chemical, Japan) that was glued to a small glass stage (the height of 2.5 mm; 2 mm in diameter) with epoxy was coated with 0.1% poly-l-lysine following the method described by Oestreicher et al. (2015). 3 μL of 0.02% glutaraldehyde was incubated on the poly-l-lysine coated mica/glass stages for 12 min, then washed 3 times using 20 μL of Milli-Q water. While keeping the stages wet, the bacterial cell suspension was applied to the mica disc by placing several micro-liters on the stage. The stage was incubated at 55°C in the light in a moist chamber to allow the bacterial cells to attach to the mica. After 1h incubation, the stage was rinsed by applying several microliters of PE medium, then wicked off, and a second rinse this with 60 μl of media. Finally, the glass stage containing the mica disc and bacterial sample was adhered to the HS-AFM scanner using nail polish.

2) High-speed atomic force microscopic (AFM) imaging

AFM imaging was performed using a custom built HS-AFM (Uchihashi et al., 2012) in tapping mode using a large scanner (6 μm × 6 μm). During imaging, the cells were kept at room temperature and imaged in PE medium. For imaging, silicon-nitride cantilever (BL-AC7DS-KU4, Olympus, Tokyo, Japan) was used at a resonant frequency of approximately 600-800 kHz. The cantilever was modified by growing a 1–2 μm long carbon tip at the end of the probe using electron beam deposition, followed by etching with an argon–oxygen plasma to sharpen the tip (Yamamoto et al., 2010).

3) Processing and analyzing HS-AFM images

Images were captured using Igor software v.6.3.4.0 (Lake Oswego, Oregon, USA), with Eagle package v.2.5.1. Images were Gaussian filtered (σ=0.5) converted to bitmap files using Kodec software v.4.4.7.39 (Kanazawa, Japan). Spatial measurements and the cross-sections of the profiles were also conducted using Kodec software.

Results

Analysis of cell-surface movement

Almost all gliding bacteria should have moving apparatus on their cell surface to move on solid surface (Spormann, 1999). In order to capture the movement of the cell-surface of the filaments, I applied glass beads (1.32 μm of median size) coated with poly-L-lysine. Microscopic observation successfully detected that glass beads moved on the cell surface along the long axis of the filaments as shown in Fig. 2-1. Speed of the movement of beads was determined to be 2.70±0.33SD μm/sec (n=16). The speed was comparable with the gliding speed of filaments, i.e., 2-3 μm/sec (shown in Chapter 3). This result suggests that the cell-surface movement propelling glass bead would be the direct driving force of multicellular filaments and enable the filaments to move straightforward.

As shown in Figure. 2-1, the beads occasionally changed the moving direction. In this observation shown in Figure. 2-1, the bead moved upward 2.9 μm in 1.5 sec, paused for 1.0 sec and then reversed the direction to go downward 2.9 μm in 1.0 sec. After that, the bead moved upward again. These changes were repeatedly observed. Beads attached to the filaments moved 2 to 5 μm in one direction and then reversed the direction. It showed that the cell-surface movements were bidirectional. Such back and

forth movements of the beads within limited area were repeatedly observed, and the moving distance was 2.5-6.5 μm (n=18).

Electron microscopic observation of C. aggregans cells

In order to observe the cell surface morphology, bacterial cells coated with a thin layer (30 nm) of osmium were observed using scanning electron microscopy (SEM). Although filaments of *C. aggregans* could be imaged, no obvious structures were observed (Fig. 2-2a). As shown in Figure. 2-2b, the cell surface was rather smooth and no characteristic structures were observed on the cell surface.

I also tried to characterize the cell morphology with a negative staining method. Filamentous cells stained by 5% ammonium molybdate were observed using transmission electron microscopy (TEM) (Fig. 2-3). Before staining, cells were incubated on a carbon grid at 55°C in the light for 10 min, to allow the bacteria to attach to the carbon film on grid and glide onto the surface. As shown in Figure. 2-3a, septa between adjacent cells within a filament were clearly observed. The cells of *C. aggregans* strain NBF were 0.4 to 0.5 μm wide, and the length of individual cells ranged from 2 to 7 μm. In my observation, two characteristic morphological features were observed; 1) fibers growing from cell surfaces (Fig. 2-3b) and 2) many linear

structures that run on cell-surface (Fig. 2-3c). The fibers were frequently found around

the septa as shown in Figure. 2-3a and 2-3b. The length of the fiber was diverse, and

ranged up to 4 μm; the width was 5.06±0.45SD nm (n=31). The fiber seemed to be

flexible, and sometimes bundled with other fibers. Some fibers lay along cells. On the

other hand, abundant slimy secretion product was not observed around cells. Focusing

on cell surface, the stripe patterns as shown in Figure. 2-3b were constantly observed.

The pattern should reflect the heterogeneity of cell surface, i.e. lineal darker area had a

feature that was easy to be stained. However, it is unclear that the heterogeneity was

attributed to the topographic feature of the cell surface, such as convex running on the

cell surface.

High-speed atomic force microscopic observation of cell surface

The cell-surface topography was observed by high-speed atomic force

microscopy (HS-AFM). HS-AFM is able to image soft biological matter, such as living

cells, in the physiological conditions (Oestreicher et al. 2015). Intact cells, i.e.

un-stained, un-fixed, and un-dried cells, could be observed by HS-AFM. While imaging,

bacterial cells were in growth media at room temperature. In this condition, cells should

be living but un-motile. Figure. 2-4a shows the cell surface topography imaged by

scanning at a size of 500 × 500 nm and a frame rate of 1990 ms/frame. The cell lies from the bottom left to the top right on the figure, and linear structures that run on cell-surface along the long axis of the cell were observed. To observe detailed structure of the cell surface, nanostructure was captured by scanning the cell at a size of 200 × 200 nm and a frame rate of 1010 ms/frame (Fig. 2-4b). The linear structures was clearly observed. The lineal brighter areas that represent higher topography were arrayed along the long axis of cell, and the darker areas represent lower topography were arrayed between the brighter areas. This characteristic topographic feature can be seen in Figure. 2-3c, that shows cross-section of the surface, where the line in Figure. 2-4b represents the location of the cross-section (Fig. 2-4c). The difference of height between the lineal brightness area and the neighbor darker area was approximately 3.3 nm. The distance between higher peak was 28-31 nm. These results indicates that cell surface of *C. aggregans* was not smooth, but linear convex structures on cell surface along long axis. The linear structures on negative stained cell-surface should be attributed to this topographic asperity.

Discussion

This study successfully detected the cell-surface movements of *C. aggregans*. The cell-surface movements propelled the glass beads along the long axis of the cells, and speed of the bead movements was quite similar to that of the gliding movements. Thus, the cell-surface movement can be hypothesized to be the direct driving force of multicellular filaments and enable the filaments to move straightforward.

A similar cell surface movement to the one observed in this study has been reported for a filamentous bacterium, *Flexibacter polymorphus* in 1988 (Ridgway and Lewin, 1988). However, possible organelles which mediated these cell surface movements remained an utter mystery. Recently, a unique movement of a cell-surface protein encoded by *sprB*, was identified in a unicellular gliding bacterium, *Flavobacterium johnsoniae* (Nakane et al., 2013). However, no *sprB*-related gene sequence has been found in *C. aggregans* genome (Klatt et al., 2007).

The cell-surface movement shown in Fig. 2-1 indicated the existence of moving component on the cell-surface that was able to attach poly-L-lysine coated beads. Although any structures like SprB that was observed in *F. johnsonie* as 150-nm-long fiber (Nakane et al., 2013) was not observed by TEM imaging of negative stained cells of *C. aggregans*, long flexible fibers growing from cell surfaces were

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observed (Fig. 2-3a and b). The fibers were up to 4 μm in length, and were approximately 5 nm in diameter. They were frequently grown from around cell poles. These fibers were not observed by AFM since AFM could not detect the structure with high flexibility. These fibers detected by TEM were quite similar to that of type 4 pili (T4P), which are known to be typically 5-7 nm in diameter and several μm in length (John 2002). This flexible fiber has been known to mediate "twitching motility" (John 2002). Gliding bacteria smoothly move and travel for long distance, whereas twitching bacteria move in a somewhat jerky fashion on solid surface. T4P located at cell poles attach a solid surface, retract, and pull the cell. T4P are mainly composed of a small protein subunit, usually termed PilA or pilin, and retraction of T4P is performed by decomposition of pilin in retraction ATPase termed PilT. The homologous sequences of pilin gene, *PilA*, and the retraction ATPase, *PilT*, have been found in *C. aggregans* genome (Klatt et al., 2007). As shown in Fig. 2-1, the beads propelled by cell-surface movement moved back and forth. The cell-surface movement was possibly mediated by bipolar pili retraction on cell surface (Fig. 2-5), i.e., a glass bead attached to cell-surface is pulled to a direction by retraction of pili in a pole, and pili in opposite pole extend; and then the glass bead moves to opposite direction when the polarity of pili extension-retraction reverses.

The linear structures that run on the cell surface was successfully detected by both TEM imaging of negatively stained cells and AFM imaging of native cells (Fig. 2-3c and Fig. 2-4). The linear structures were attributed to topographic feature of the cell surface, i.e. about 3.3 nm height and 30 nm width of convex structures that run on the cell surface along the long axis. I couldn't observe the structures on cell surface by SEM, possibly because the cell surfaces were coated with 30-nm-thickness layer of osmium at SEM imaging and the nanostructures were shaded. Linear structure on cell surface has been reported in gliding filamentous cyanobacteria, *Oscillatoria* sp. strain A2 (Read et. al., 2007). In this cyanobacterium, the linear structure was attributed to 25- to 30-nm-diameter fibrils that situated between the peptidoglycan and the outer membrane with helical array. The linear structures on cell surface would help that the filament moves straightforward with revolving. However, the linear convex structure observed in *C. aggregans* was not with obvious helical array. Thus, the linear structure would help a filament to move straightly without revolving. Moreover the liner convex structure may help to arrangement of pili on cell-surface (Fig. 2-5).

In this study, I found the cell surface movements as well as possible structures related to gliding motility of *C. aggregans*, T4P-like pilus and linear convex structures on cell surface. I propose that cell-surface movement mediated by pilus retraction is the

direct force of gliding as a possible model of gliding machinery of *C. aggregans*.

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Figures and legends
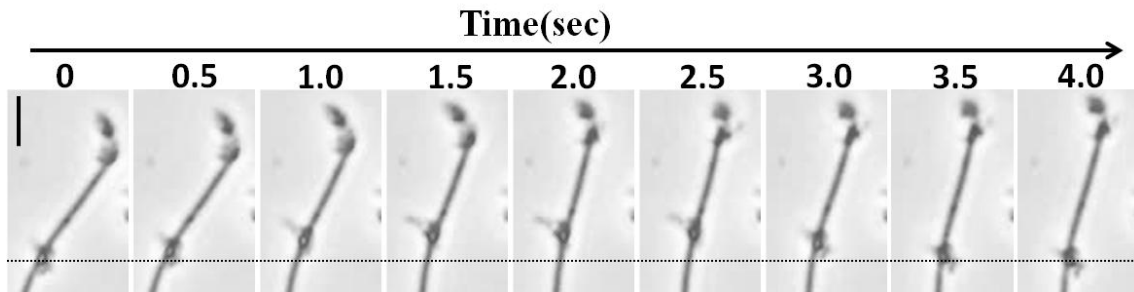
Fig. 2-1. Sequential micrographs taken at 0.5 sec intervals showing the reversal

movement of a glass bead on a filament. Scale bar, 5μm.

(a)

(b)
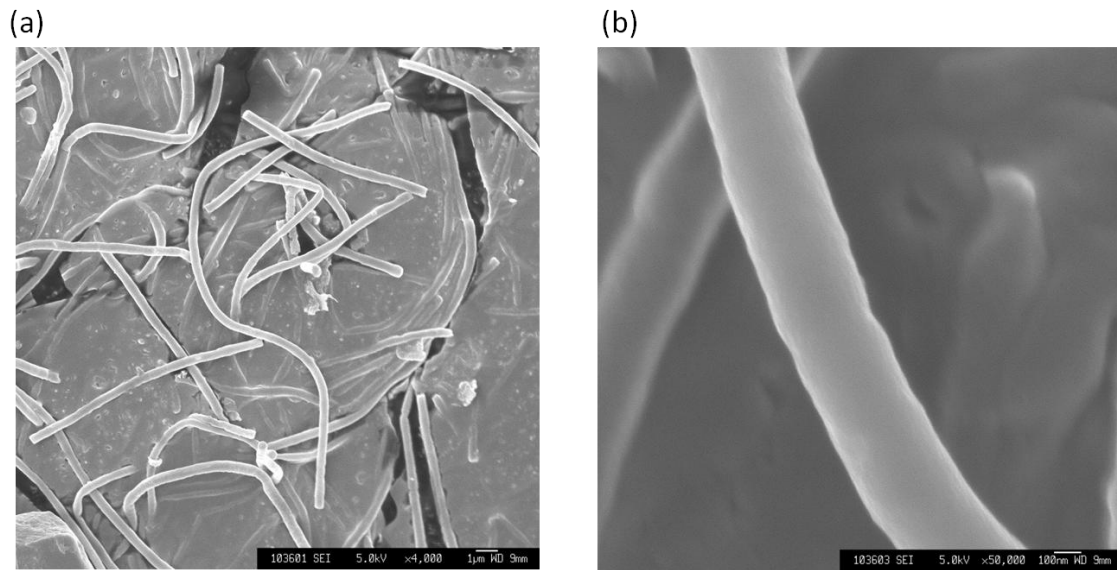
Fig. 2-2. Scanning electron microscopic images of *C. aggregans* cells coated with a thin

layer (30 nm) of osmium. (a) 4,000 magnification. (b) 50,000 magnification.
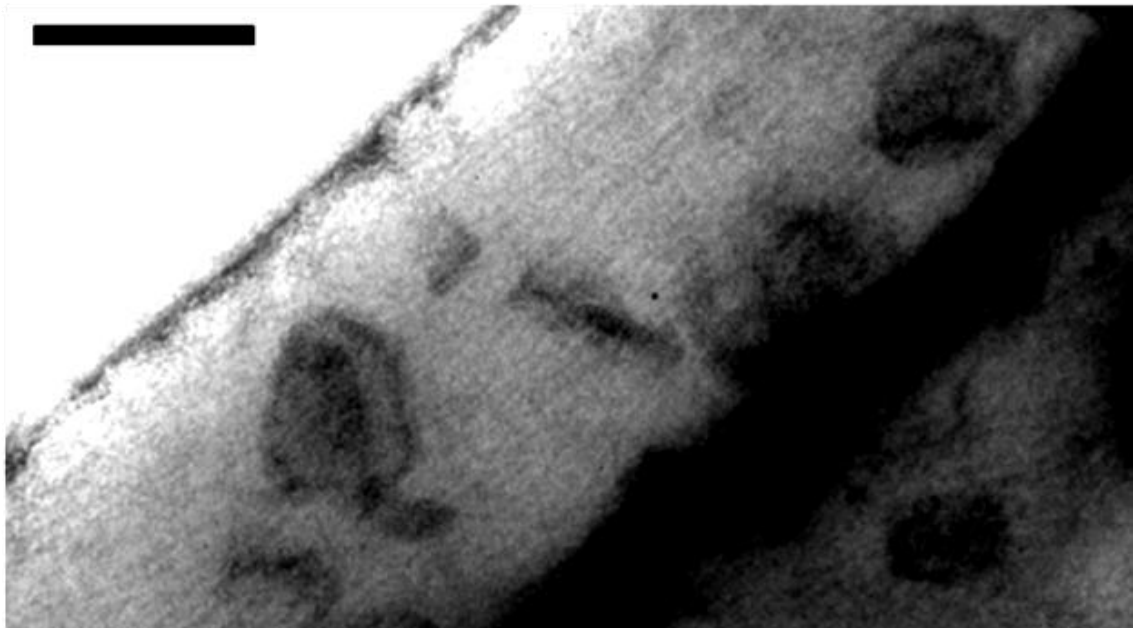
(a)

Fig. 2-3. Transmission electron microscopic images of negative stained cells of *C. aggregans*. (a) 8,000 magnification. Scale bar, 2μm. (b) 30,000 magnification. Scale bar, 200nm. (c) 50,000 magnification. Scale bar, 200nm. Arrows indicates some of pili.
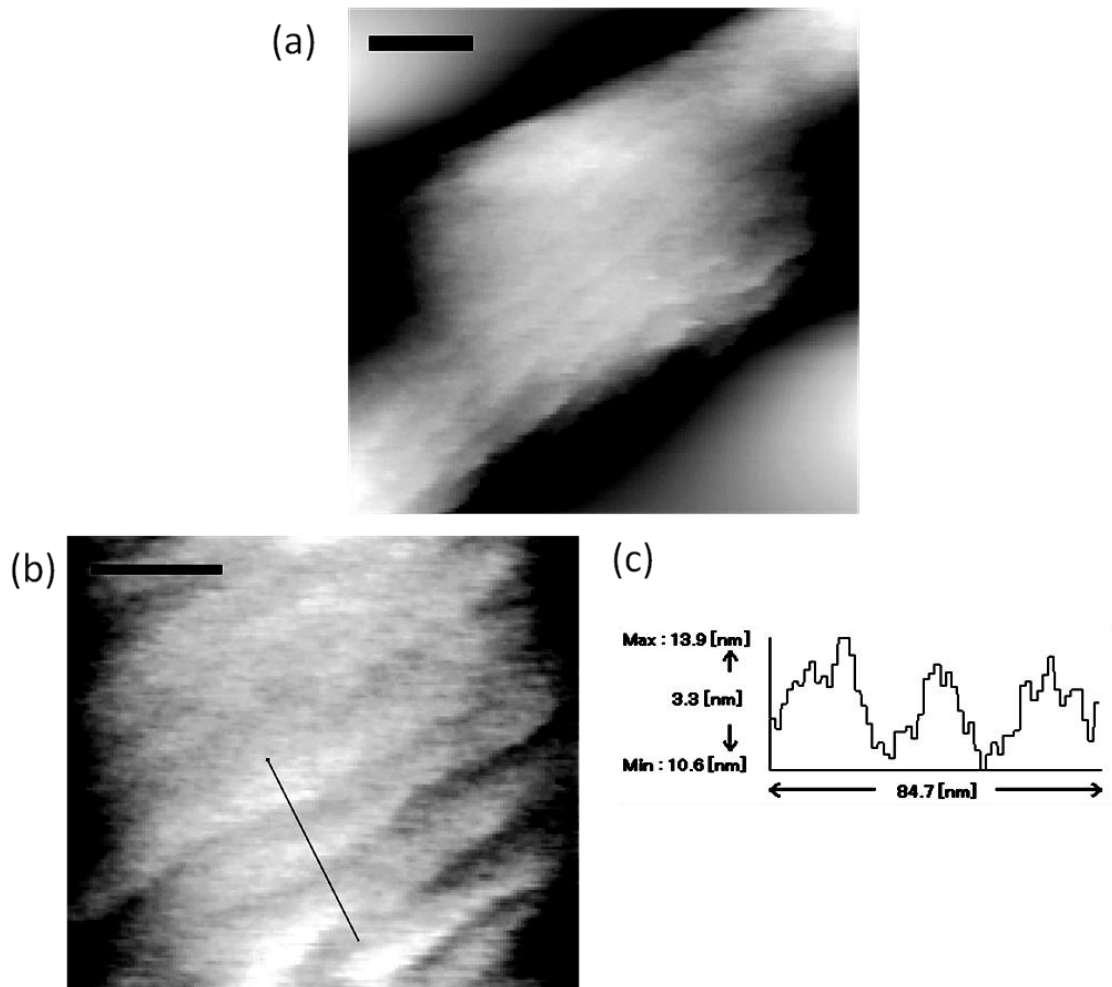
(b)
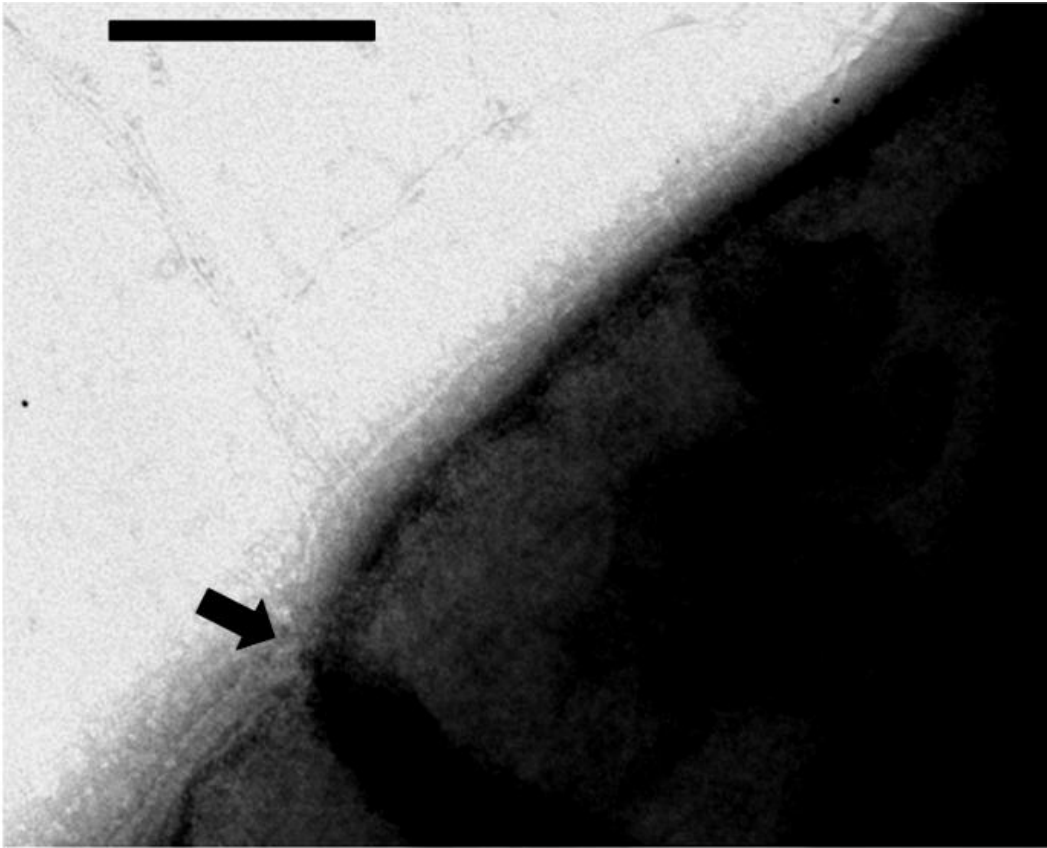
(c)

Fig. 2-4. High-speed atomic force microscopic images of *C. aggregans* cell (a and b)

and cross sectional topography of cell-surface (c). (a) A scanning area of 500 × 500

nm and a frame rate of 1990 ms/frame. Scale bar, 100nm. (b) A scanning area of 200 ×

200 nm and a frame rate of 1010 ms/frame. Scale bar, 50nm. The line in panel (b)

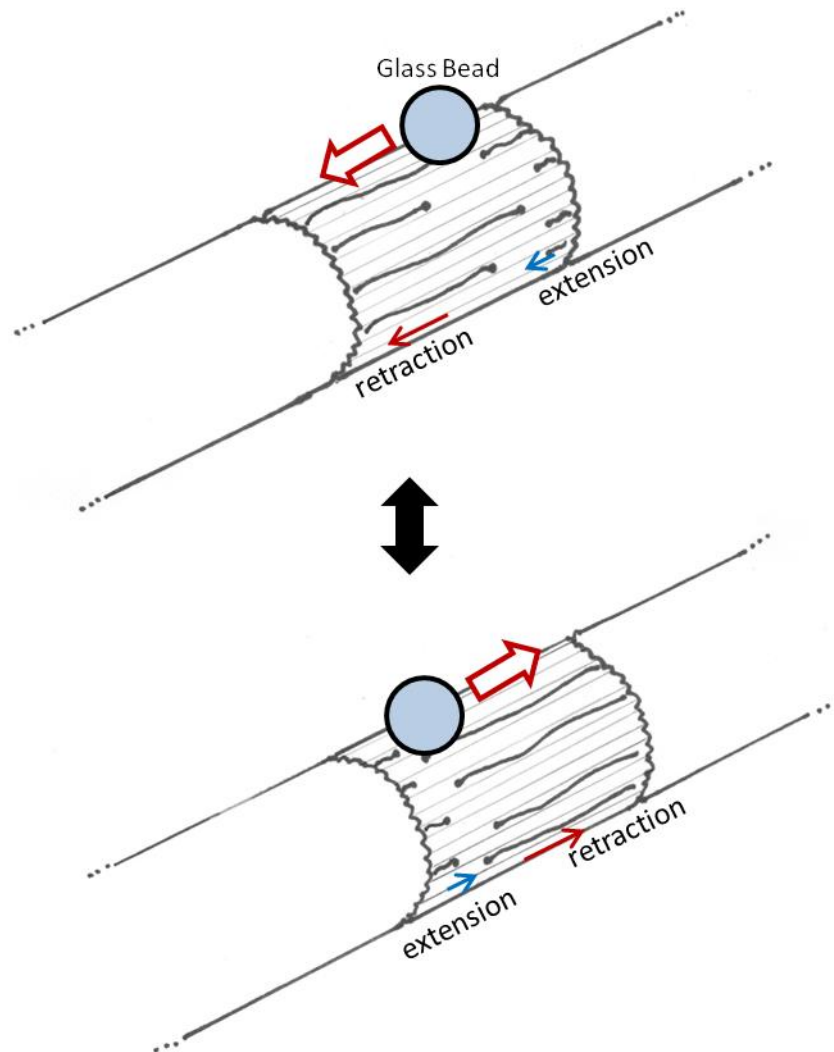represents the location of the cross-section shown in (c).

Fig. 2-5. Schematic diagram of a possible mechanism of cell-surface movement. The

cell surface movement may be mediated by bipolar pili extension and retraction, e.g., a

glass bead attached to cell-surface is pulled by bi-directional pili retraction.

Chapter 3

Determination of moving direction in a multicellular

filamentous bacterium *Chloroflexus aggregans*

Abstract

Chloroflexus aggregans is an unbranched multicellular filamentous bacterium having the ability of gliding motility. The filament moves straightforward at a constant rate, approximately 3 μm/sec on solid surface and occasionally reverses the moving direction. In this study, we analyzed movements of glass beads on the cell-surface. Microscopic analyses found that the cell-surface movements were confined to a cell of the filament, and each cell independently moved and reversed the direction. To understand how the cellular movements determine the moving direction of the filament, we proposed a discrete-time stochastic model; sum of the directions of the cellular movements determines the moving direction of the filament only when the filament pauses, and after moving, the filament keeps the same directional movement until all the cells pause and/or move in the opposite direction. Monte Carlo simulation of this model showed that reversal frequency of longer filaments was relatively fixed to be low, but the frequency of shorter filaments varied widely. This simulation result appropriately explained the experimental observations. This study proposed the relevant mechanism adequately describing the motility of the multicellular filament in *C. aggregans*

Introduction

Chloroflexus aggregans is a filamentous anoxygenic photosynthetic bacterium in the phylum *Chloroflexi*. The cells (1.0-1.5 μm wide; 2-6 μm long) form unbranched multicellular filaments of indefinite length, that the length excess of 400 μm in some filaments. The filamentous morphology is widely found in the deeply branching phylum *Chloroflexi* (Hanada, 2014). Gliding motility, non-flagellated movement on solid surface, has been reported for phototrophic groups (the families *Chloroflexaceae*, *Roseiflexaceae* and *Oscillochloridaceae*) and some species of non-phototrophic bacteria in this phylum (Hanada, 2014). *C. aggregans* isolated from terrestrial hot springs moved straightforward along long axis of the filament at speeds of 1 - 3 μm/sec at 55°C (Hanada et al., 1995), which is more than 10 times faster than other bacteria belonging to the phylum *Chloroflexi*.

Although gliding motility of unicellular bacteria such as *Flavobacterium*, *Myxococcus* and *Mycoplasma* has been well studied (Shrivastava et al., 2015; Nan et al., 2014; Miyata, 2010), only few reports dealt with gliding of multicellular filamentous bacteria. As shown in Chapter 2, the moving force for gliding should be cell-surface movement along long axis of a filament in *C. aggregans*. However, no experimental results have obviously given a comprehensive explanation for gliding characteristics of

filamentous bacteria, *e.g.*, how they move straightforward, how they change the moving direction and whether cells in a filament are cooperative or independent.

In this study, I examined the directions and distances of cell-surface movements within a filament of *C. aggregans* and clarified the effect of the filament length on gliding movements. Finally, using a Monte Carlo simulation, I propose a relevant mechanism, that explain how multicellular filamentous bacteria move straightforward at constant rate and occasionally change the moving direction.

Materials and methods

Organism and growth conditions

    Chlorolfexus aggregans strain NBF used for this investigation was previously isolated from Nakabusa Hot Springs in Japan (Morohoshi et al., 2015). The cells were anaerobically grown in PE medium (Hanada et al., 1995) at 55°C under incandescent light.

Microscopic analysis of the gliding motility

    C. aggregans cells were collected in exponential growth phase by centrifugation at 4,000 × g for 2 min and re-suspended in fresh PE medium. The cell suspension was poured into a 'tunnel' (16-mm interior width, 18-mm length, and 1-mm wall thickness) constructed by taping a coverslip to a glass side using double-sided tape, following the method described by Uenoyama et al. 2004. Cell behavior was observed under a phase-contrast microscope (AXIO Imager A2, Carl Zeiss, Oberkochen, Germany) equipped with a heating stage at 55°C (BLAST Kanagawa, Japan). Micrographes were recorded with a DP73 camera (Olympus, Tokyo, Japan) and CellSens standard software (Olympus).

Analysis of cell-surface movements

Micro-glass beads (1.32 μm of median size, Nihon Horo Yuyaku, Tokyo, Japan) were suspended in 0.05% poly-L-lysine solution, and incubated at room temperature for 1 hour. The beads were washed three times with distilled water and suspended in PE medium. Culture solution of *C. aggregans* was mixed with the beads solution and centrifuged (4,000 × g for 2 min). The precipitate including the bacterial cells and beads was gently re-suspended in a fresh PE medium. The movements of glass beads attached on bacterial cells were observed with a microscope as described above.

Stochastic modeling of the movements of multicellular filaments

In order to simulate how movement of cells affected moving direction of the filament, discrete-time stochastic models were proposed. For simplicity, we described the movement in one spatial dimension, i.e., right or left, where the directional movements were described as +1 (right) and -1 (left) and 0 meant the pausing of the movement. Cell index (k) within a multicellular filament is 1, 2, 3, … k_{max}, (i.e., k_{max} is the cell number of a filament), and the moving direction of each cell at time t is $C_k(t)$ (here, t=0,1,2,...).

In model A, the moving direction of a filament is determined by a majority of

all the cells composed of the filament in every time. Thus, the gliding direction of a

filament at time t, $G(t)$, is defined as below;

$$G(t) = \begin{cases} +1, & (R(t) > L(t)) \\ -1, & (R(t) < L(t)) \\ 0, & (R(t) = L(t)) \end{cases} \tag{1}$$

where $R(t)$ and $L(t)$ are the number of cells moving right (+1) and left (-1) within a

filament at time t, respectively.

In model B, $G(t)$ refers to the preceding movement, $G(t-1)$. If the filament

pauses at time t-1, i.e., $G(t-1) = 0$, the moving direction of a filament at time t, $G(t)$

is determined by a majority of all the cells composed of the filament as defined with

equation (1). On the other hand, if the filament moves at time t-1, i.e., $G(t-1) \neq 0$,

$G(t)$ is defined as described below;

$$G(t) = \begin{cases} G(t-1), & (N(C_k(t) = G(t-1)) > 0) \\ 0, & (\text{otherwise}) \end{cases} \tag{2}$$

where $N(C_k(t) = G(t-1))$ is the number of cells at time t moving to the same

direction as the moving direction of the filament at time t-1.

If $N\big(C_k(t) = G(t-1)\big) > 0$, i.e., more than one cell moves toward the same

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direction with the preceding direction of the filament, the filament keeps a unidirectional movement. If no cell moves toward the same direction with the preceding direction of the filament, i.e., all the cells at time t are pausing and/or moving in the opposite direction to the moving direction of the filament, the filament stops to move, i.e., $G(t) = 0$. After that the moving direction at the next time $G(t+1)$ is determined again by equation (1) as described above.

We computed $G(t)$ for filaments composing of various numbers of cells ($k_{max} = 25,30,...110$) by Monte Carlo method (Matlab software, Mathworks, Natick, MA, USA). At time 0, moving direction of cells, $C_k(0)$ and the filament, $G(0)$ is set as 0. Each cell in the filament moves to right or left with equal probability, and otherwise pauses. Therefore, $C_k(t)$ takes values in +1, -1 and 0 with the following probabilities (P);

$$P\{C_k(t) = +1\} = P\{C_k(t) = -1\} = A \tag{3}$$

$$P\{C_k(t) = 0\} = 1 - 2A \tag{4}$$

where a stochastic parameter A is a constant varied between 0 and 0.5.

Reversal frequency of gliding movement is defined as the number of sign change of $G(t)$, i.e., +1 to -1 or -1 to +1, over simulation time, t_{max}.

Results

Analysis of direction of cell-surface movement

As described in Chapter 2, when glass beads (1.32 µm median size) coated with poly-L-lysine were applied to cell suspension, the beads attached on cell surfaces moved along long axis of the filaments. The beads oscillated back and forth within 2.5-6.5 µm of the distance. The cell-surface movements were likely confined to a section of the filament. One unit of the section was closely related to the cell, since moving ranges of the beads were within the length of each cell. During further microscopic analyses of glass beads movements, I found that two beads attached on different positions of one filament moved in separate directions (Fig. 3-1). These results suggested that each cell in a filament moves individually and the cellular movements are independent from each other.

Motility characteristics of C. aggregans

Movements of the multicellular filaments at 55˚C were observed with a microscope. The filaments moved straightforward along the long axis (Fig. 3-2a). Speed of the gliding movements was determined to be 2.58±0.59 µm/sec (n=63, 36-450 µm length of the filaments), which was not affected by the filament length. No definite

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acceleration and slowdown were observed in the moving filaments. The filaments occasionally reversed the direction without pausing as shown in Figure. 3-2a. The numbers of times that the filaments changed the direction were counted during the observation and the reversal frequency per 10 min was determined. The results obtained for each length of the filaments were plotted in Figure. 3-2b. The reversal frequencies of shorter filaments (n=45, 36-100 μm) were 1-16/10 min, and those of longer filaments (n=32, 100-450 μm) were 0-5/10 min. Reversal frequencies of longer filaments tended to be lower and less variable compared to shorter filaments.

How do C. aggregans *filaments change the direction?*

As shown above, the segmented cell surface movements seem to determine the movements of *C. aggregans* filaments. The moving direction of the filaments may be decided by a majority of the movements of individual cells. In order to understand how cellular movements affect the moving direction of the filaments, I tested two discrete-time stochastic models to simulate the movements of the filaments composed of multiple cellular units. In the simulation, I set the movements of individual cells that switch stochastically and independently.

Firstly, I tested a simple model; sum of the direction of all the cells always

determines the direction of the filaments (model A, see Materials and Methods). I

simulated the relationship between reversal frequency of filaments and the cell number

of each filament using Monte Carlo method followed by equation (1). Fig. 3-3a shows

the results showing reversal frequencies of filaments, calculated 200 times for each

filament. The reversal frequency tended to increase by increase in the number of cells,

i.e., filament length, and variability of reversal frequency for each filament was not

affected by the filament length. These results did not match the experimental

observation shown in Figure.3- 2.

Then, I tested another model; sum of the direction of all the cells determines

the direction of the filaments only when the filaments pause, and after moving, the

filament keeps the same directional movement until all the cells pause and/or move in

the opposite direction (model B, see Materials and Methods). This model also means

that separate directions of the cellular movements did not have a tug of war, i.e., the

filament keeps moving in a unidirectional manner as long as at least one cell moves to

the direction and the moving direction is decided only when the filament pauses. Figure.

3-4b shows that reversal frequency of longer filaments is relatively fixed to be low, but

the frequency of shorter filaments varies widely. The revised model appropriately

matched the experimental observation (Fig. 3-2). Longer filaments infrequently got the

chance that all the cells in the filament stop the cell-surface movement, but the reversal

frequency of shorter filaments was stochastically changing.

Discussion

This study successfully found that the cellular movements within a filament were independent each other, and filament lengths affected the reversal frequency of gliding movements.

The beads movement in *C. aggregans* was sectioned in the filaments, and each section was individually moving and changing the moving direction as shown in Figure. 3-1. My observation indicated that a section was consistent with a single cell. A cell-surface movement has been reported for a filamentous gliding bacterium, *Flexibacter polymorphus* (Ridgway and Lewin, 1988). Although the cell-surface movements in *F. polymorphus* was not sectioned with each cell, the movements were directionally independent within a filament.

My primary question was how the multicellular filaments move at constant speed and reverse the moving direction without long pausing; and how the moving characteristics of each cell, i.e., independently moving and stochastically changing the moving direction, made the moving characteristics of the filaments. In the study of *F. polymorphus*, Ridgway and Lewin (1988) described that the relationship between directions of cell-surface movements and that of filamentous movement was mystery. However, stochastic modeling and the experiments shown in Figure. 3-1 successfully

led me to obtain a probable answer. It is noteworthy that the moving direction of the filament is determined by a majority of the moving into directions of cells in the filament only when the filament pauses (Fig. 3-5a). Importantly, cells moving separate directions in a filament do not have a tug of war while the filament is moving (Fig. 3-5b). As the results, the filament moved at constant speed. It is likely that movement of the filament does not allow cells with cell-surface movement in the opposite direction to generate force for gliding. Moreover, our model appropriately explained the smooth reversal movement shown in Figure. 3-1a; just before the gliding filament paused, the cell-surface movement of most cells paused or moved to the opposite direction (Fig. 3-5c). This is the mechanism firstly proposed to adequately describe the motility of multicellular filaments.

Ridgway and Lewin (1988) reported that a multicellular filamentous bacterium, *F. polymorphus* showed a similar gliding motility with that of *C. aggregans*, i.e., the smooth reversal movement and the relationship between reversal frequency and filament length. The mechanism of direction determination of gliding proposed using stochastic simulation is probably applicable to *F. polymorphus*.

Reversal frequency is an important factor affecting population behavior such as taxis and formation of cell aggregate (Daniel and Lawrence, 1998). My model proposed

here indicates that increase in time or probability for pausing of cell-surface movement, i.e., $P\{C_k(t) = 0\}$ is increased, results in an increase in the reversal frequency of the filaments (Fig. 3-4). *C. aggregans* makes cell aggregates in liquid culture through the gliding motility on the other filaments without tight adhesion between filaments (Hanada et al., 1995). Morohoshi et al. have reported that an extracellular protease promoted the aggregating motility in *C. aggregans* (Morohoshi et al., 2015a). Morohoshi (2015b) also reported that the activity was controlled by reversal frequency depending on the signal product promoted by the protease. These behaviors of multicellular filamentous bacteria can be simply controlled by cell-surface movement of cells composing the filaments.

The model proposed in this study successfully explains motility of filamentous organism, i.e., how a filament moves straightforward and changes the moving direction by independent movements of cells within a filament. This simple mechanism found in the deep-branching bacterium is rather ancestral, and can be commonly employedto many organisms and systems composing multiple units.

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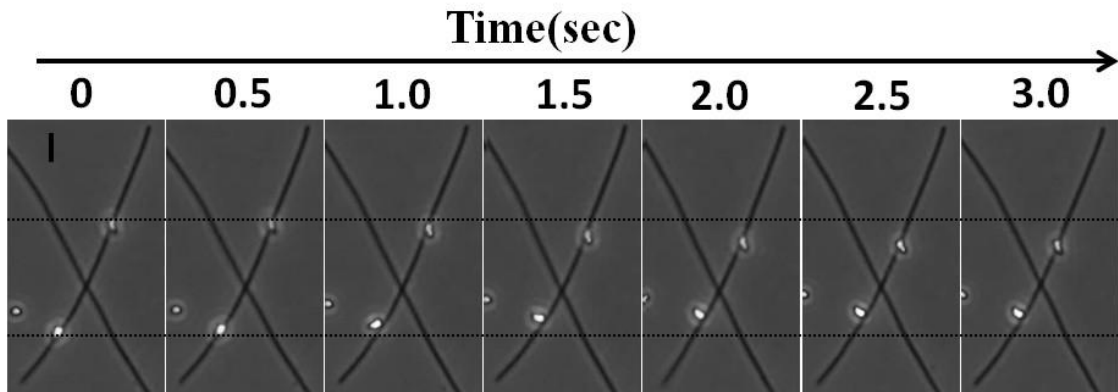
Fig. 3-1. Sequential micrographs taken at 0.5 sec intervals showing the separate

movement of two glass beads on a filament. Scale bar, 5μm.

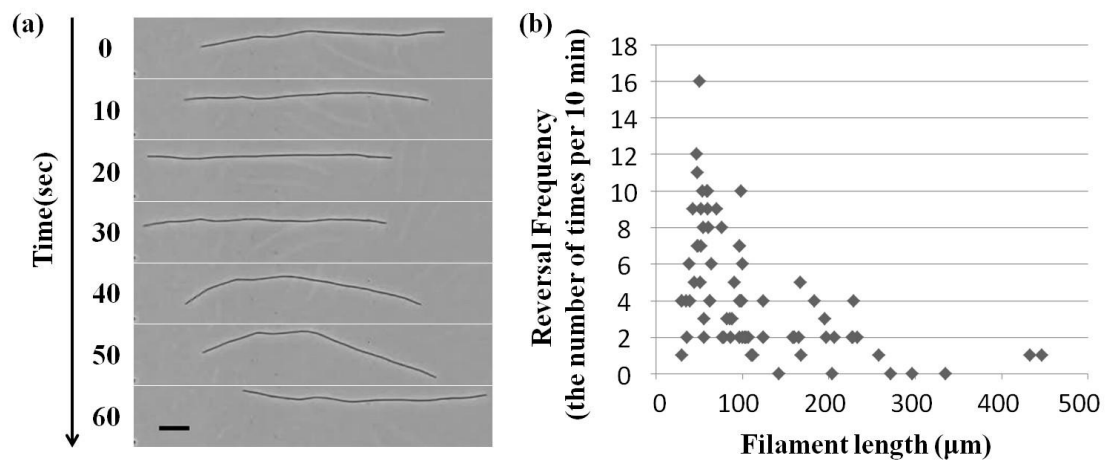Fig. 3-2. Gliding motility of a filament of *C. aggregans*. (a) Micrographs taken at 10 sec intervals showing a reversal in the direction of gliding. Scale bar, 10 μm. (b) Relationship between reversal frequency (the number of times of the reversal movement per 10 min) and the filament length. Results obtained from 77 filaments (36 – 450 μm length) were plotted.
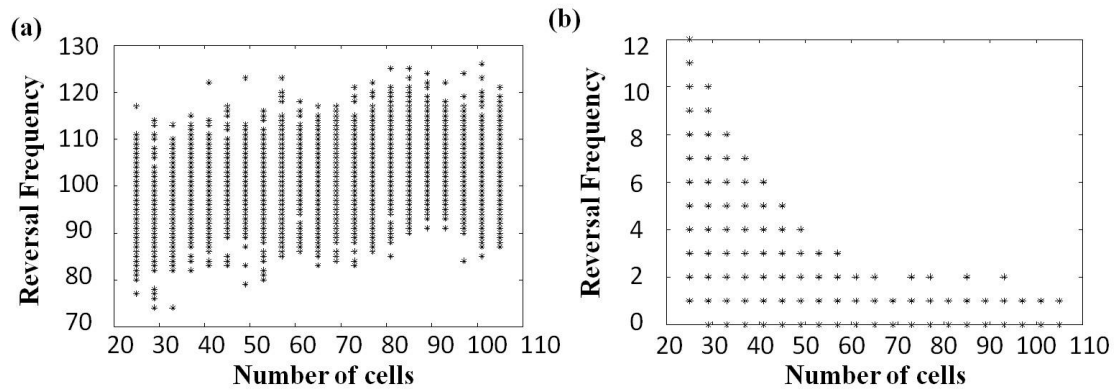
Fig. 3-3. Simulation results of relationship between reversal frequency and the cell

number of each filament. The number of reversal frequency in a simulation run time

(t_{max}=1000) was calculated for the filaments with 25-105 cells. (a) and (b) are the results

of 200 times simulation for each filament using simulation model A and B, respectively

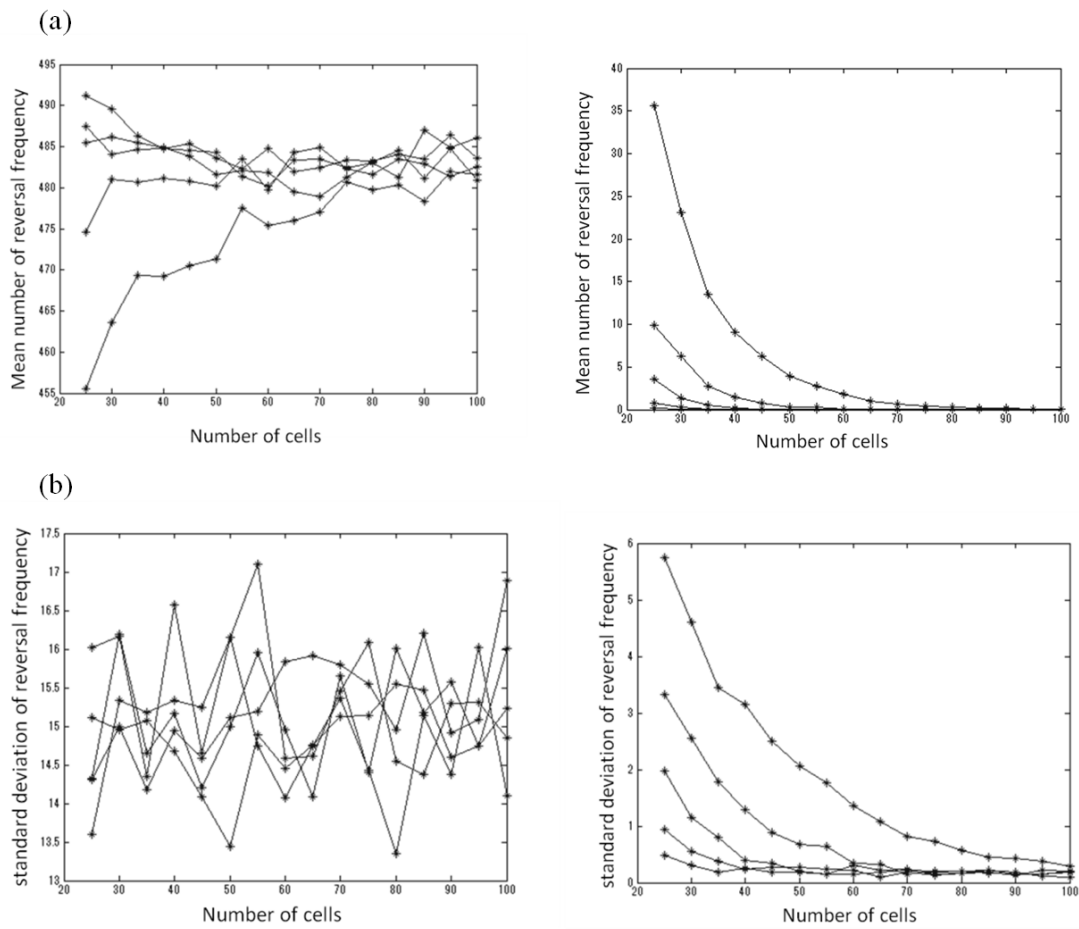(see Materials and Methods). Stochastic parameter A, 0.125.

(a)

(b)

Fig. 3-4

The dependency of pausing frequency of each cell-surface movement to reversal

frequency in Model A and B. (a) Simulation results of relationship between mean

number of reversal frequency and number of cells consisting a filament under various

stochastic parameter A (A=0.3, 0.25, 0.2, 0.15, 0.1) in Model A (left) and Model B

(right). (b) Simulation results of relationship between the standard deviations (SD) of

reversal frequency and number of cells in Model A (left) and Model B (right). In model

A, mean number of reversal frequency tend to increased depending on value of A in

lower number of cells ((a) left), but the SD didn't depend on both the number of cells and value of A ((b) left). In model B, although mean number of reversal frequency of each number of cells increased depending on value of A, the tendency that both mean number and SD of reversal frequency decrease depending on number of cells was independent from value of A ((a)right and (b) right).
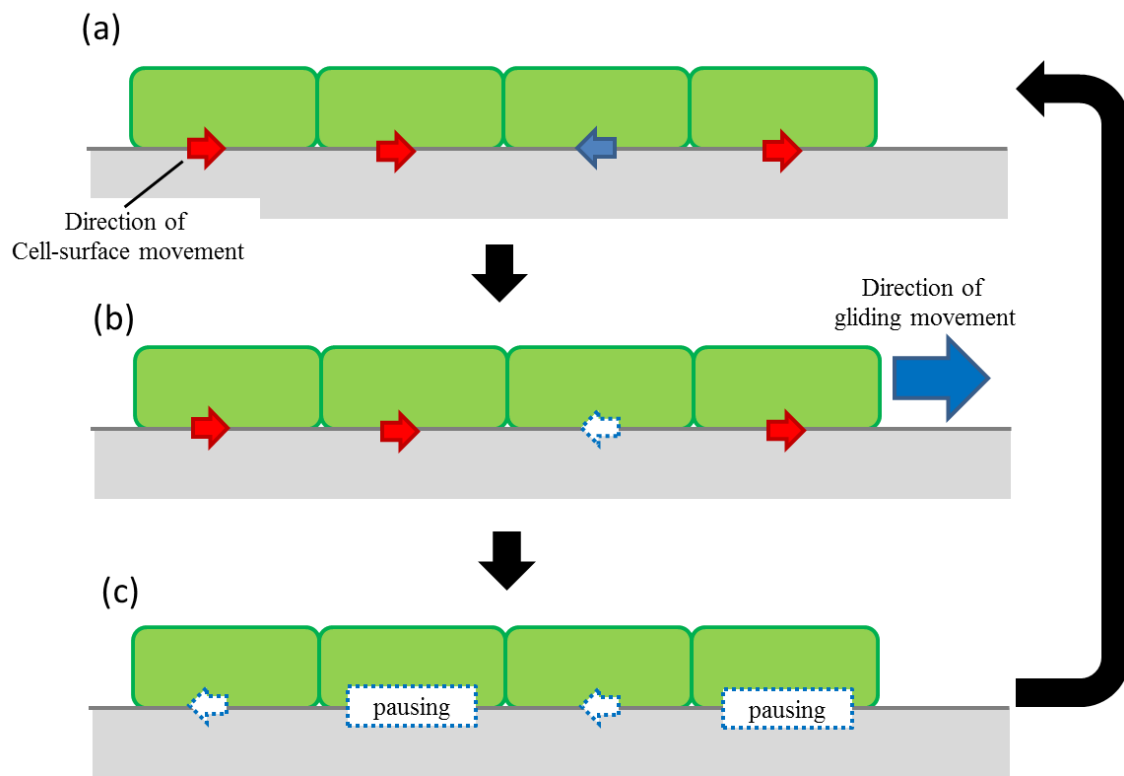
Fig. 3-5 Schematic diagram of a possible determination mechanism of moving direction in multicellular filamentous bacteria. (a) When the filament is pausing, the gliding direction of the filament is determined by a majority vote among the cell-surface movements. (b) The direction of gliding movement is kept while the filament is moving. (c) The filament pauses when the cell-surface movement of most cells paused or moved to the opposite direction (Fig. 3-5c).

Total discussion

In this study, I found that a filamentous gliding bacterium *C. aggregans* moved toward semi-aerobic environment in the dark (Chapter 1). I also detected segmented cell-surface movements and possible cell-surface structures related to the gliding motility of *C. aggregans* (Chapter 2). Finally, the gliding motility was successfully simulated by a stochastic model, in which a filament moves straightforward and changes the moving direction by independent movements of cells within a filament (Chapter 3).

Bacterial motility has been recognized as a strategy to respond and adapt to environmental changes (Bray, 2001). I found that aerotactic behavior that cells of *C. aggregans* moved toward semi-aerobic environment (Fig. 1-1, Fig. 1-2 and Fig. 1-4). This ability of aerotaxis should be advantageous for survive of *C. aggregans*, because oxygen is essential for aerobic respiration in the dark but should be rather toxic for anaerobic photosynthesis in the light. Chemotactic behaviors including aerotaxis are generally controlled by the frequency of changing direction of moving (Barry et al., 1999). My stochastic modeling indicated that the reversal frequency of multicellular filamentous bacteria can be controlled by the frequency of pausing of cell-surface movement, i.e. increase in the pausing frequency of each cell results in an increase in

the reversal frequency of the filament (Fig. 3-4). *C. aggregans* cells may change the

pausing frequency according to intra- or/and extra-cellular oxidative states. This is a

reasonable system since bacteria can adapt to environments without wasting a lot of

energy.

Reversal frequencies of longer filaments were lower and less variable

compared to shorter filaments (Fig. 3-2b). The simulation results also show that the

reversal frequency decreases as the filament length increases (Fig. 3-3b). These results

suggest that properties of the gliding motility are strongly coupled with the multicellular

filamentous morphology in *C. aggregans*.

The stochastic modeling and simulation of the gliding motility of *C. aggregans*

gave the following two important suggestions; (1) moving direction of the filament is

determined by a majority of the moving directions of cells in the filament only when the

filament pauses; (2) separate directions of the cellular movements do not have a tug of

war while the filament is moving. How are these indications about gliding system

accordance with a possible moving machinery, i.e., pilus retraction on cell surface

coordinated the cell-surface movements (Fig. 2-5 and Fig. 3-5) ? Here, this study

proposes a model of gliding mechanism in *C. aggregans* that explains the results of the

multi-scale analysis of the motility comprehensively; the moving direction of a filament

is decided by majority vote among separate directions of pili on each cell-surfaces when the filament pauses, and, at the moment, pili of "defeated direction" would once disengage a hand from the solid surface, i.e. the pili would be removed or jolt out of alignment from the contact area between cells and solid surface. The filament keeps moving in a unidirectional manner as long as at least one cell moves to the direction, because the pili of "defeat direction" don't play a role in the gliding movement. The pili of "defeat direction" would be more difficult to grip the solid surface and/or more easier to lose contact with the surface than that of "winner direction". It is a possible reason why the pili of "defeat direction" would be less tense than the pili of "winner direction" that pull the filament.

This is the first study that shed light on the mysterious gliding mechanism of *C. aggregans* belonging to a deep-branching phylum *Chloroflexi*. Gliding motility has been found in various bacteria, and the mechanisms are diverse. This study would be helpful to understand the evolution and diversity of gliding mechanism. Moreover, this study reported a system that the individual cell behaviors control the well-ordered multicellular behavior without cooperative function such as signal transduction among cells. The simple system like this might be employed widely in bacterial world.

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