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論文内容要約

Chapter 1. General Introduction

Bacteria are the unicellular microscopic organisms, 0.5-1 micrometers in diameter and a few micrometers in length, not visible with the naked eyes. Bacteria can be found virtually everywhere on the earth. They are in the soil, air, water, rock, oceans and even arctic snow. Motile bacteria have a filamentous motile organ called flagellum, which consists of a basal body (flagella motor), hook and filament. The bacterial flagellar motor is a rotary motor embedded in the cytoplasmic membrane, which is powered by an electrochemical potential difference generated across the cytoplasmic membrane. The ability to move (motility) is very essential for any biological life, even for human beings, to maintain their life activities. Bacteria also use motility for searching appropriate places where cells colonize (soil, water, the interior of animal bodies). Bacteria move for searching foods and nutrients, to complete their life cycles in various environmental situations, migrating from one host to another or one part of body to another. The ability to change the moving direction is important for bacteria to move toward nutrients and get away from toxins. For surviving, it is important to avoid unfavorable habitat and to find favorable environments containing positive stimuli, such as a plentiful nutrients, light and oxygen. This behavioral system is called “taxis”; especially, the response against chemical substrates is called “chemotaxis”. Motility and chemotaxis are considered to be two important virulent factors for pathogenic bacteria. The bacterial motility and flagellar function are important for medical scientists as motility is known to be related with the establishment of infection and the virulence of pathogens. For engineers, the bacterial motility is a fine model of nano/micro machineries. Physicists would be interested in their well harmonized movement in the microscopic world dominated by thermal fluctuation. The present study was conducted at the perspective of biophysics to understand how bacteria achieve a stochastic migration toward a place where more nutrients are present **(Chapter 2)** and by what the motility is driven **(Chapter 3)**. Here I focused on the motility of *Leptospira*, a member of helically shaped bacteria called spirochetes. Most of motile bacteria have flagella at the cell

exterior, but spirochetes including *Leptospira* have flagella at the cell interior. Hiding flagellum within the body is an admirable idea to prevent the biological actuator from damages but making the motility mechanism complicated. I developed a novel observation technique to quantitatively analyze the motility of *Leptospira* (Chapter 2).

Chapter 2. Analysis of chemotactic behavior of Leptospira by microscopic agar drop assay

Chemotaxis is a behavioral property that bacteria detect nutrients or toxins through sensors and stochastically migrate toward favorable places for growing. It is known that bacteria having multiple external flagella regulate the ratio of 'tumbling' to smooth swimming for chemotaxis. The tumbling is caused by reversal of flagellar rotation from counterclockwise to clockwise, allowing cells to stop swimming transiently and change the swimming direction randomly. The tumbling frequency is decreased with increases in the concentration gradient of nutrients in externally flagellated bacteria, achieving gathering of cells at higher concentrations of nutrients. Genetic and structural studies have shown that *Leptospira* also possesses similar chemotaxis system with externally flagellated bacteria, it remains unknown how *Leptospira* cells control their behavior in the presence of nutrients or toxins.

To investigate chemotaxis of *Leptospira*, I developed a novel method to analyze the chemotaxis of *Leptospira* under a microscope. The developed assay called microscopic agar-drop assay (MAA), where a droplet of agar containing attractants or repellents is placed at the center of a flow chamber, allowed us to observe the real-time movement of bacterial cells: attractive migration to or escaping from chemical compounds diffusing from the agar drop. MAA is a simple method, with being made up of a glass slide, cover slip and double-sided tape; no requirement of incubation before observation; completion of experiment for less than 20 min. MAA showed that *Leptospira* cells gradually accumulated around an agar drop containing an attractant such as glucose and sucrose. Accumulation of cells around the agar drop was dependent on the concentration gradients of attractant. MAA also showed negative responses of *Leptospira* to fructose and mannitol.

I found that *Leptospira* cells frequently altered the shape of cell-body and displayed vigorous "rotation". During rotation, the cells translated neither forward nor backward and slightly change the direction of the cell axis, which was similar with the tumbling in externally flagellated bacteria. We revealed that the tumbling frequency is decreased at a higher glucose concentration suggesting that sensing an attractive chemical allows these cells to swim more smoothly. On the basis of experimental results, I proposed a model of chemotaxis mechanism that *Leptospira* switches the motion between 'translation' and 'tumbling' with the cell-body transformation and controlling the ratio of translation to tumbling is responsible for biased

migration toward nutrients.

Because *Leptospira* is known to possess genes of chemotaxis receptors called MCPs (Methyl accepting chemotactic proteins) in their genome, MCPs are thought to be primarily involved in the attractive or repellent chemotaxis to sugars. Although many attractants are nutrient for bacteria, *Leptospira* cannot use sugars as a nutrient. A phosphotransferase system (PTS) is known as a MCP-independent pathway causing chemotaxis and contributing to various regulatory functions in bacteria. In *Leptospira*, sugars might induce a chemotactic response through PTS, which may also be associated with some metabolic activities.

I validated that MAA can also be used to evaluate chemotaxis of the externally flagellated bacteria *Salmonella enterica*: *Salmonella* cells displayed attractive and repellent responses against serine and nickel, respectively. Thus, MAA can be used for studying chemotaxis of not only *Leptospira* but also other motile bacteria.

Chapter 3. Ion selectivity of the Leptospira flagella motor

The flagellum is composed of a filament, functioning as a propeller, and a rotary motor called flagellar motor. The flagellar motor consists of a rotor and a stator like an artificial motor and converts the flux of ion through the stator into rotation. A kind of ion coupling with rotation varies depending on species of bacteria, for example, proton (H^+) is used by *Escherichia coli* and *Salmonella*, and sodium ion (Na^+) is used by the marine bacteria *Vibrio* and alkalophilic bacteria. The coupling ion of the *Leptospira* flagellar motor had remained unclear. In this study, I analyzed the motility of *Leptospira* under various conditions of pH and salt concentrations.

Leptospira cells displayed active motility in acidic to alkaline pH conditions and the motility increased with pH. Swimming speeds and membrane voltages were increased with an elevation of extracellular pH. In *E. coli*, the loss of ΔpH due to increase in the extracellular pH can be compensated by increase in membrane voltage. It has known that the contribution of two energetic components such as ion gradients and membrane voltage are not equal for the rotation of flagella motor. The contribution of membrane voltage might be larger than that of ΔpH in *Leptospira* and showing faster motility at pH 9.0. When a protonophore, CCCP was added to media, inhibiting H^+ translocation through stators, cells completely lost motility at pH 7.0 indicating that H^+ is the major driving force for the *Leptospira* at neutral pH. At pH 9.0, cells were not completely stopping their motility and showed very slow movement although membrane voltage measures at pH 8.5 were approximately the same level as that at pH 7.0. This result raises a possibility that other ions besides H^+ contribute to the motility.

I found that the motility was enhanced by the addition of Na^+, suggesting that Na^+ is also involved in

the motility of *Leptospira*. Cells exhibited vigorous motility in the absence of Na^+, indicating that Na^+ is not essential for motility. Probably, H^+ is used preferentially over a wide pH range and that Na^+ is used secondarily. Since the enhancement of motility by the addition of Na^+ was observed at both neutral and alkaline conditions, explicit switching between H^+ and Na^+ depending on pH does not appear to occur. In alkaline conditions, Na^+ might compensate the reduction in contribution of H^+.

Genome sequence analysis has shown that *Leptospira biflexa*, a species used in this study, contains two *motA* genes (*motA-1* (LEPBI_I0196) and *motA-2* (LEPBI_I0136)) and two *motB* genes (*motB-1* (LEPBI_I0197) and *motB-2* (LEPBI_I2845). *motA-1* and *motB-1* genes are connecting with each other (partially overlapped) as with genes of H^+-type stator in *E. coli* and *Salmonella*, whereas *motA-2* and *motB-2* are separated. It is feasible that MotA-1/MotB-1 and MotA-2/MotB-2 are the H^+-type and the Na^+-type stators, respectively. However, sequences of transmembrane domains of *motB-1* and *motB-2* in *Leptospira biflexa* are almost the same, though the transmembrane domain of MotB is thought to play a key role for ion selectivity. Thus, functional differentiation between MotA-1/MotB-1 and MotA-2/MotB-2 may not be expected. These genetic insights raise a hypothesis that both H^+ and Na^+ are translocated through the same stator unit, either MotA-1/MotB-1 or MotA-2/MotB-2. In this study, I only cloned *motA-1* and *motB-1* genes into a plasmid, showing that both H^+ and Na^+ are transported through a single set of stator MotA-1/MotB-1. Therefore, an idea that only MotA-1/MotB-1 function in *Leptospira* flagellar system can explain all of the present experimental results. However, it is difficult to completely deny the possibility that MotA-2 and MotB-2 is also constitutively expressed. To clear this matter, further experiment using gene-knockout mutants on *motA-1/motB-1* and *motA-2/motB-2* are required. This is the first report showing the dual-ion system of the *Leptospira* flagellar motor.

Chapter 4. Conclusion

In this study, I developed a novel motility-analysis method MAA for real-time observation of *Leptospira* chemotaxis. This method will be widely used for motility experiments and contribute to deeper understanding of the ecology of microbes. I found that *Leptospira* uses both H^+ and Na^+ for flagellar rotation. The pliable selectivity of the input energy can be advantageous for bacteria to survive in various environments. Results obtained in my study surely promoted deeper understanding of the motility mechanism of *Leptospira*, but raising many new questions.

How do *Leptospira* cells actually change the swimming direction by tumbling behavior? Although the tumbling in peritrichous bacteria can randomly change the axis of cell body at the extent enough to change swimming direction (≈ several-ten degrees), the tumbling of *Leptospira* seemed to change the cell axis and

swimming angle just a little degrees (data not shown). Although it is difficult to accurately analyze the swimming trajectory of *Leptospira* due to frequent dynamic transformation of the ends of cell body, further detailed observation of tumbling behavior would achieve tangible argument and understanding on chemotaxis.

Concerning the rotation of *Leptospira* flagella, I would like to figure out the energy-conversion mechanism in the future. It is thought that torque generated by the *Leptospira* flagellar motor is about twice as large as that of *E. coli* flagellar motor. What is the mechanism producing such large torque? The energy-conversion efficiency in *Leptospira* motor is higher? To give answer to these questions, a measurement of the amount of input energy is required. In my experiments, I succeeded in measuring intracellular pH and membrane voltage using fluorescent dyes, but measuring resolution was not enough to discuss the results quantitatively. Combining analysis of motility (output) with higher temporal/spatial resolution and measurements of energetic parameters (input) should be helpful to understand the operating principles of the flagellar motor dynamics and to develop future nanomachines.