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Bacterial sheet-powered rotation of a micro-object

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ABSTRACT

Recently, there have been numerous reports of the use of microbes as bio-actuators. In particular, bacteria are one of the promising candidates for bioactuator development, because of which they have garnered a lot of interest lately. In this paper, we developed a new system for the transport of a micro-object by using surface swarming *Vibrio alginolyticus*, a marine bacterium, and achieved high-speed movement of the micro-object. First, we confirmed if YM19 and YM19ΔcheY, two genetic variant strains of *V. alginolyticus* that are appropriate for surface swarming, were able to transport microbeads. We found that the transport speed of YM19ΔcheY was higher than that of YM19, and this confirmed high validity of genetic modification. We further tried to control the movement by constructing a microwall and succeeded in causing the rotational transport of the micro-object. Finally, we demonstrated microgear rotation with a rotational speed that was about 7 rpm, thus confirming the effectiveness of our proposed method.

1. Introduction

Micro-nano-robots and bio-MEMS are well-studied systems with extensive applications in medicine [1]. To engineer a micro-nano-robot that can move in blood vessels, it is necessary to engineer an actuator for driving it. A micro-nano-actuator is also required as a built-in mechanical factor in bio-MEMS. The components are required for engineering a micro-nano-actuator include (i) a mechanical and electrical factor, (ii) a biological macromolecule including protein [2], and (iii) a living organism itself [3–12]. Biological macromolecules and living organisms work as very small and highly effective actuators, because they utilize chemical energy instead of electromotive force. Thus, these bio-actuators are being touted as "Next Generation Motivity." Despite the advances in the field of bio-actuator research, many problems remain unsolved. For instances, the biological function of the living component is not well characterized in some cases. Therefore, the development of an efficient actuator design is important.

Several recent reports describe the construction of bio-actuators with microbes. More specifically, the rotation of microgears by bacteria is one area of extensive research. Some bacteria are flagellated and can swim in aqueous environments by using the flagella as screws. Flagellar rotation is brought about by a flagellar motor, which is embedded in the bacterial cell membrane [13–16]. Hiratsuka et al. [10] reported the rotation of microgear by using mobile Mycoplasma, where the bacteria attached to a microgear directory were pushing the microgear. Further, Sokolov et al. [11] and Leonardo et al. [12] reported that randomly swimming bacteria could rotate a gear with an asymmetrical shape (ratchet shape). However, the rotational speed of the microgear in this case was low (1–2 rpm). Therefore, an improvement of rotational speed was desired.

In this study, we have used *Vibrio alginolyticus*, a gram-negative marine bacterium. *V. alginolyticus* is suitable for use in a bioactuator because this species has some distinct advantages. First, *V. alginolyticus* has a specific motile mode known as "Surface swarming". This bacteria have two types of flagella, the polar flagellum and

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the lateral flagella. The bacteria, rod-shaped swimmer cell, usually swim in aqueous environments using polar flagellum, which is produced constitutively; however, when the bacterial cells are introduced in high-viscosity environments such as animal surfaces, they switch to surface swarming [17]. Surface swarming cells become elongated in shape and generate new peritrichous flagella (lateral flagella) for surface swarming and thus move over the surface. This movement resembles social motility. Actually surface swarming cells can move on 1.3% agar plate, whereas swimming cells could not move in this condition. In this motile mode, the characteristics of the motile bacterial sheet (carpet) [18] are different from those of swimming [11,12] and gliding bacterial cells [9,10], making it a good candidate for use in a bioactuator. We expected some advantages in this motile mode. (1) It can easily provide a high density motile cells (The space is filled with almost 100% bacteria). (2) High torque is expected because the number of flagella for one cell also increased. (3) High torque is expected by social motility like movement of surface swarming cells. Hence, in our present study, we have used bacterial cells in this motile mode as a high torque actuator. Second, genetically engineered strains have been well established in this species such that the inherent properties of the cells can be exploited (genetic engineering was not used in the previous report [10–12]). At first, the basic property of transporting the microbeads using the surface swarming property of *V. alginolyticus* was analyzed. Moreover, to achieve high-speed rotation of the micro-object, to a level that could be achieved for micro-gears, we aimed to regulate the direction of the transport and to apply this motion to rotate the micro-object.

2. Materials and methods

2.1. Bacterial strains, growth conditions and media

V. alginolyticus strains used are a polar flagellum-defective mutant, YM19 (Pof^{-} Laf^{+}), YM19ΔcheY, in which the *cheY* gene is deleted, was constructed by an in-frame deletion in the *cheY* gene with homologous recombination. Mean swimming speeds of YM19 and YM19ΔcheY cells are 15.2 ± 4.1 (μm/s) and 23.1 ± 6.3 (μm/s), respectively. *V. alginolyticus* cells were cultured at 30 °C in VC medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.2% glucose), VPG medium [1% polypeptone, 0.4% K_2HPO_4, 3% NaCl, 0.5% (w/v) glycerol].

2.2. Transport of micro-object

The conditions and procedures used in the experiment performed for transporting the microbeads were as follows: 1.3% VPG agar in 9-cm culture plates. The bacteria were cultured in VC medium for 14 h at 30 °C. Two microliters of bacteria cultured overnight was inoculated at the center of the VPG plates. Drying of the plate such that the liquid was no longer visible was taken as confirmation that the liquid drop with bacteria had seeped into the plate. These plates were incubated for a few minutes at 30 °C. After the short incubation, 2 μl of VPG solution with 10 μm (diameter) microbeads was introduced at the spot of bacterial cell inoculation on the plate. The plates were then incubated for 4 h at 30 °C, after which the bacteria formed a sheet. The behaviors of the bacterial cells and the microbeads were observed by optical microscopy.

2.3. Fabrication of a ratchet-shaped microwall

To regulate rational direction, we fabricated a ratchet-shaped microwall. The method for the fabrication of the microwall was as described below. The pattern was etched on a silicon wafer using a focused ion beam (FIB) and this wafer was used as a template. Polydimethylsiloxane (PDMS) was poured on to the silicon wafer, which was then incubated under isothermal conditions to rid the wafer of the template. The fabricated microwall was further observed by optical and laser microscopy (Fig. 1A and B).

2.4. Regulation of transport using the ratchet-shaped microwall

A culture of the YM19ΔcheY strain was prepared by incubation at 30 °C for 14 h. Two microliters of this bacterial culture was inoculated onto a 1.3% VPG agar plate and the plates were incubated at 30 °C for 4 h. After incubation, as in case of the experiment with microbeads, microbeads of 1.3 μm diameter were introduced to facilitate observation of the bacterial sheet. The fabricated ratchet-shape microwall was then introduced onto the bacterial sheet and observed using optical microscopy.

2.5. Microwall fabrication by photolithography

The silicon wafer was cleaned by ultrasonic treatment and then spin coated with SU-8 3010 at 3300 rpm. The spin-coated wafer was then exposed to a micropattern generator (μ-PG), and the patterned wafer was developed and used as the template. PDMS was then poured onto the silicon wafer, which was then incubated in an isothermal incubator to rid the wafer of the template. After sufficient hardening of the PDMS, we rid the template from the PDMS. The fabricated microwall was observed by optical and laser microscopy (Fig. 1C).

2.6. Microgear fabrication by photolithography

A silicon wafer was cleaned by ultrasonic treatment and then spin-coated with AZ5214E at 500 rpm. A second spin coating was performed with SU-8 3005 at 7000 rpm. The spin-coated wafer was then exposed to a μ-PG and developed after patterning. The fabricated microgear was then observed by optical and laser microscopy (Fig. 1D).

2.7. Driving the microgear into the microwall

We first prepared 9-cm 1.3% VPG agar plates, and streaked a single colony onto the plate. The plate was then incubated for 6 h at 30 °C. The microgears were picked up using manipulators and placed on the bacterial sheet. The microwall was placed on the microgear, and the setup was observed by optical microscopy.

3. Results and discussion

3.1. Transport of the micro-object by the bacterial sheet

Well-established genetic engineering techniques are in place for *V. alginolyticus* such that the bacterial cells are made amenable to modifications of their morphological properties. In this study, we used a mutant strain of *V. alginolyticus*, YM19, which constitutively generates lateral flagella for surface swarming. Furthermore, we detected the *cheY* gene, which controls the chemotaxis system, for generating highly motile bacterial cells [19]. The chemotaxis genes regulate flagellar motor rotation based on environmental cues from outside the bacterial cell. The CheY protein, which is a response regulator in the chemotaxis system, can regulate flagellar rotation by associating with the flagellar motor directly. In surface swarming of *V. alginolyticus*, CheY slows down the rotational speed of the flagellar motor. Therefore, disruption mutants of the *cheY* gene move very fast because of the constitutively high-speed rotation of the flagellar motor (Fig. S1). In this case, bacterial behavior cannot be controlled by environmental stimuli (chemical, thermal, etc.), although the transport ability increases due to high-speed movement. To examine whether surface swarming of

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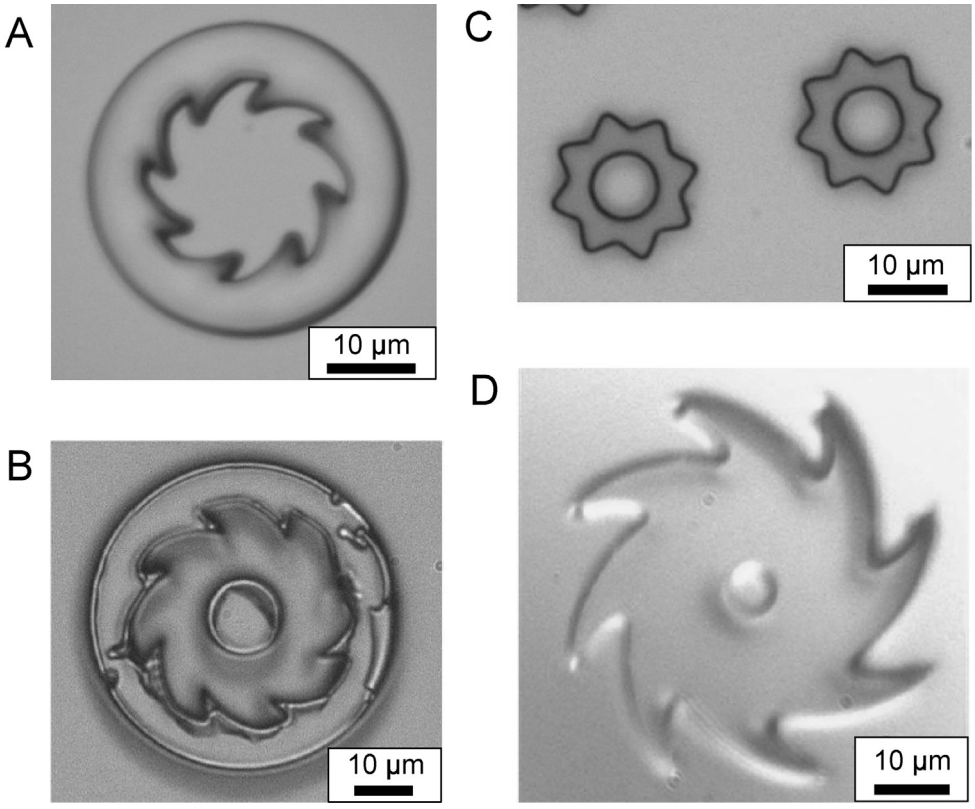

Fig. 1. Optical microscopy of fabricated ratchet-shaped microwall. Microwall for beads (A), microwall with pillar (B), microgear (C) and microwall for gear (D).

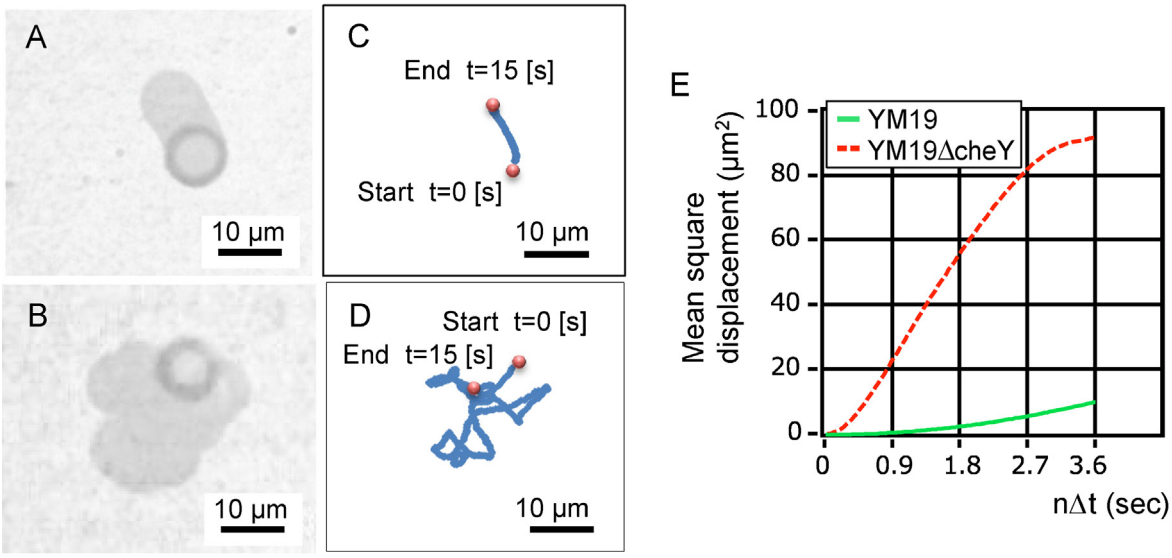

Fig. 2. Trajectory of microbead moved by YM19 (A), (B) and YM19ΔcheY (C), (D) for 15 s. Motion of microbeads is shown as the shaded region in (A) and (C). The trajectory of the center position is shown in (B) and (D). Mean-square-displacements of the beads on YM19 and YM19ΔcheY are shown in (E). Solid and dashed lines indicate YM19 and YM19ΔcheY, respectively.

V. alginolyticus can aid in the transport a micro-object, we tried to transport microbeads (polystyrene) by using surface swarming of YM19 and YM19ΔcheY. Optical microscopy indicated that beads with 10 μm diameters were transported. From this observation, we confirmed the movement of microbeads on the bacterial sheets of YM19 and YM19ΔcheY. To compare the movements of the microbeads on YM19 and YM19ΔcheY, we tracked and constructed a trajectory of microbead movement. Fig. 2 indicates the result of tracing the movement for 15 s, where the motion of the microbeads is shown has been shaded (Fig. 2A and C). The trajectory of the center of mass was calculated by microbeads movement and shown in Fig. 2B and D. The trajectories of both strains seem chaotic, such that the direction of transport by the bacterial sheet can be said to be random. The area spanned by the movement of the bead with YM19ΔcheY is larger than that with YM19. Furthermore, the mean-square-displacement of the bead on YM19ΔcheY is clearly higher than that on YM19 (Fig. 2E). These results indicate that the transport speed of YM19ΔcheY is faster than that of YM19. This

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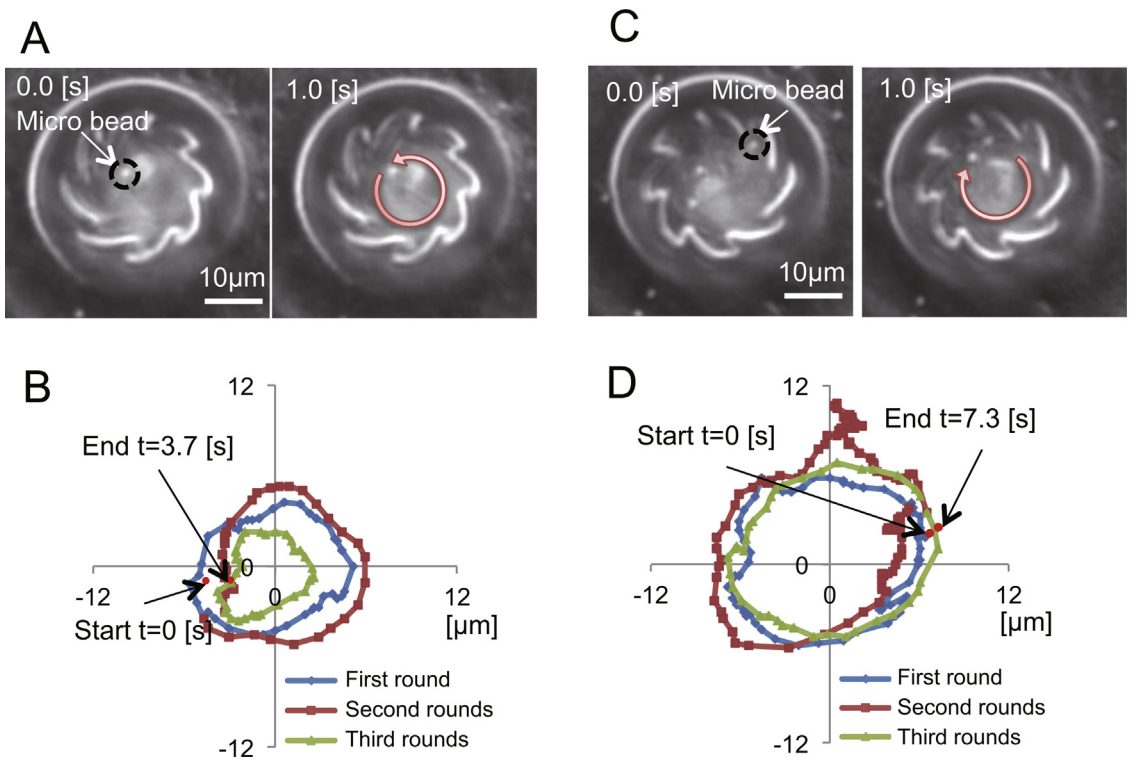

Fig. 3. Counterclockwise rotational movement of the microbeads covered by the CCW ratchet-shaped microwall (A) and its trajectory for 3 rounds (B). Clockwise rotational movement of the microbead covered by the CW ratchet-shaped microwall (C) and its trajectory for 3 rounds (D).

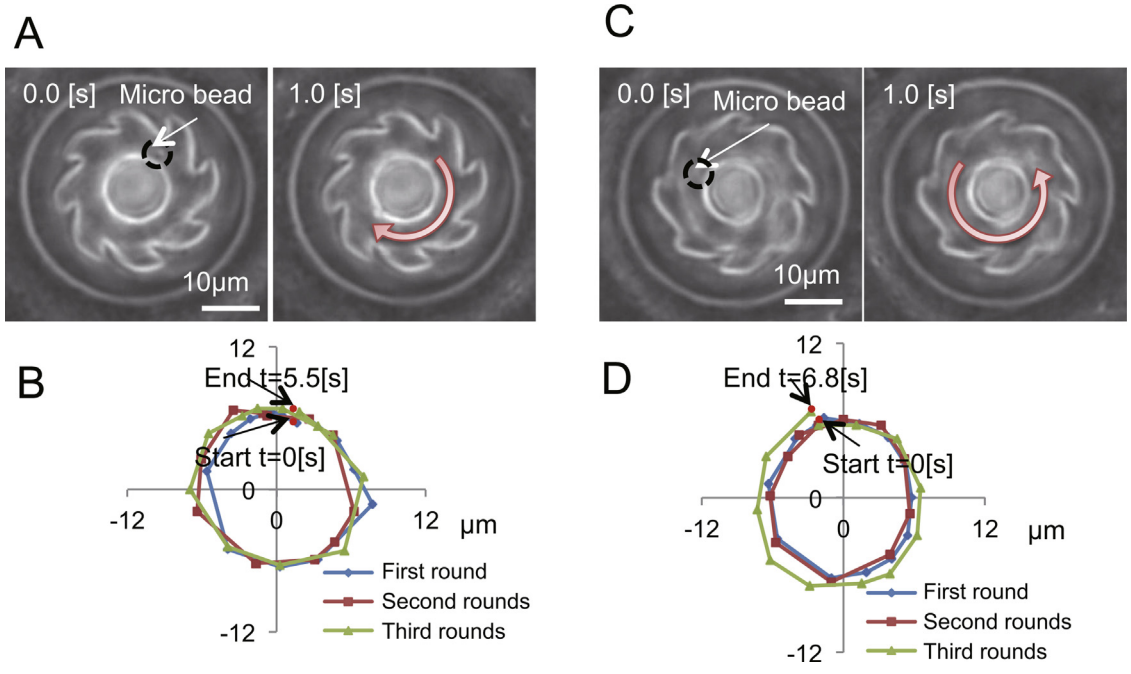

Fig. 4. Counterclockwise rotational movement of the microbeads covered by the CCW ratchet-shaped microwall with pillar (A) and its trajectory for 3 raps (B). Clockwise rotational movement of the microbead covered by the CW ratchet-shaped microwall with pillar (C) and its trajectory for 3 raps (D).

phenomenon is consistent with our hypothesis described above. The microbeads seemed to be transported by the flow caused by bacteria movement and direct pushes by bacteria. However, moving direction of object is same as bacteria motile direction. Detail generation mechanism of flow by bacteria movement is unknown. Future studies are needed to reveal that.

3.2. Regulation of the transport of microbeads by the ratchet-shaped microwall

To regulate the movement of the microbeads, an asymmetric (ratchet-shaped) microwall was used for controlling the motion of the bacterial sheet. In this experiment, we fabricated two

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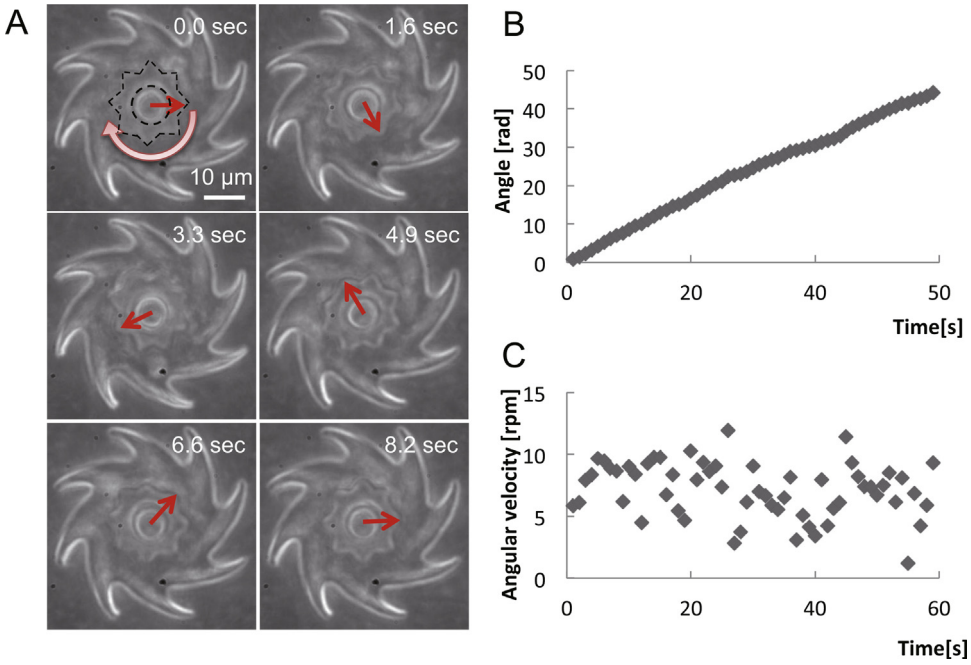

Fig. 5. Rotational movement of microgear in microwall. Results are shown as sequential images (A), the graph of the angular motions for every 1 s (B), and fluctuations in angular velocity plots (C).

differently shaped walls—one clockwise (CW) and one counter clockwise (CCW) ratchet-shaped wall. The results of the observation of the fabricated microwalls by optical microscopy show that the inner diameter of the ratchet-shape microwall was about 16 μm, the external diameter was 24 μm, and the depth was about 7.5 μm (Fig. 1A shows the result of the 7.5-μm deep CW).

As the next step, we observed the movement of the microbeads with the ratchet-shaped microwall. The results for the 7.5-μm deep wall are shown in Fig. 3. Fig. S2 shows sequential images of the movement of a single bacterium for 1.4 s. The movement of the bacterium was random; therefore, at times, the bacterium moved backwards (see Fig. S2 at 0.44 s). In this case, the bacterium was caught on to a barb, and then moved forward again (see Fig. S2 at 0.93 s), so that the movement of the bacterium was limited to a single direction.

Fig. 3A shows CCW rotation of the transported beads as sequential images in 1.0 s (Movie S1). The 1.3-μm microbeads had a CCW rotational motion, and this trajectory for 3 rounds is shown in Fig. 3B. From this result, we were able to calculate the velocity of microbead movement, and the bead rotated at 48.6 rpm on an average. Fig. 3C shows CW rotation of transported beads. The 1.3 μm microbeads had a CW rotational motion (Movie S2). From the trajectory data, we calculated the velocity of microbeads and these beads rotated at 24.7 rpm on an average (Fig. 3D). Sometimes, the microbeads crossed the center of the microwall. Therefore, the trajectory and velocity of microbeads was not stable and could not be precisely calculated.

We attempted to regulate the rotational direction of the movement of the bacteria by using the microwall. However, the rotational movement was not smooth. Microbeads were sometimes trapped in the barb. To rectify this inconsistency in rotational movement, we fabricated a center pillar in the microwall (Fig. 1B). We then observed the movement of the microbeads with the ratchet-shaped microwall with the pillar. The conditions and the procedure of the experiment were same as in the previous case. The results are shown in Fig. 4. This time the microbeads were not held up on the barb and rotated smoothly in the surroundings of the pillar. Thus, we achieved smooth rotational movement using the pillar. From this result, the CW rotation speed was 27.9 rpm on an average (Fig. 4B), and the CCW rotation speed was 34.3 rpm on an average (Fig. 4D). The difference in the CW and CCW rotation speeds was significantly reduced.

Consequently, clockwise and counterclockwise rotation was achieved. Moreover, it was confirmed that the microbeads rotated on the bacterial sheet. The fractions of the microbeads moving in the intended directions were almost 100%. The rotation continued for at least 3 h. However, the rotational speed decreased gradually. We believe that the decrease in the rotational speed is caused by the extensive bacterial proliferation near the microwall. After 3 h, there was a significant loss of space for movement.

3.3. Driving microgear

To use actuation by bacterial sheet as motor, we fabricated microgears using SU-8 and the microwall using PDMS, and tried to drive the microgear using the rotational movement of bacteria. Optical microscope image of fabricated microgear is shown in Fig. 4A. The external diameter of the microgear was 19 μm and the thickness was 2 μm. The image of the fabricated microwall, as seen under the optical microscope, is shown in Fig. 4B. The inner diameter of the microwall was 33 μm, the diameter of the pillar was 7 μm, and the depth was 10 μm.

The results are shown as sequential images in Fig. 5A (Movie S3). From this result, we could estimate the velocity of rotation of the microgear by image analysis. Fig. 5B shows a graph of the angular motions for every 1 s. This result indicates that the microgear was stably rotating. The fluctuations can be better appreciated in the angular velocity plots in Fig. 5C. The microgear showed smaller fluctuations in angular velocities, compared to that reported previously [11]. Furthermore, the fractions of the microgears moving in the intended directions were almost 100%. The ratchet-shaped microwall generated a constant movement of bacteria and regulated bacterial cell number depending on the wall size. Thus, a stable rotation of microgear was achieved.

From the calculations, the microgear was found to rotate at about 7.1 ± 2.2 rpm on an average. The rotation of the microgear

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was significantly slower than that of microbeads. The microgear become slower speed for receiving the larger viscous resistance than that of microbeads. Furthermore, in the case of microgear, for displaced rotational center during the rotation of microgear, there might be frictional resistance between the central fixed pole and hole of the gear. Therefore, the rotation of the microgear becomes slow. In this case, the linear speed of the edge is about 7.1 μm/s, whereas *V. alginolytius* could move at approximately 20 μm/s. The circumference of the microgear was about 60 μm. Thus, the microgear might rotate at a maximum speed of 20 rpm. This observation leaves a large margin of improvement with regard to microgear rotation. Improvements in the experimental conditions might facilitate faster rotation.

4. Conclusions

In the present study directed towards the application of bioactuators, we established a driving system using a bacterial sheet. First, we succeeded in controlling the rotational direction of bacteria by using a microwall. To use the bacterial sheet as a motor, we fabricated a microgear and a microwall. We succeeded in driving the microgear into the microwall. Reductions in the size of the motor toward the micro/nano-meter scale have been hindered by problems such as the reductions in the sizes of the driving forces including energy supply systems. Our proposed bio-motor is one solution to these problems. Furthermore, this system could be used for the further analysis of bacterial cell properties such as surface swarming, which are not well studied so far. Therefore, we can find interesting phenomenon, for example similar to *E. coli* guiding movement [20], from this microwall system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2015.07.071.

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Biographies

Masaru Kojima received Doctor of Science degree from Nagoya University in 2006. He had been a Research Fellow with the Department of Biology, Nagoya University in 2007, Research Fellow on Global Center of Excellence (GCOE) with the Department of Biology, Nagoya University in 2008, Assistant Professor on GCOE with the Department of Micro-Nano Systems Engineering, Nagoya University from 2008, Assistant Professor in Department of Systems Innovation, Osaka University from 2012. His main research interest is biophysics, in particular, using the biological object and proteins as bio actuator.

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Michio Homma received the Doctor of Science Doctor of Science degrees from Tokyo University in 1985. During 1985–1988, he was a Postdoctoral Associate with the Department of Molecular Biophysics Biochemistry, Yale University. During 1988–1992, he was a Lecturer with the Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, where he was promoted to an Associate Professor. During 1992–1998, he moved to the Department of Molecular Biology, Faculty of Science, Nagoya University, Nagoya, Japan, where he has been a Professor with the Division of Biological Science, Graduate School of Science. His current research interests include energy transduction and sensory transduction in bacteria, and a special emphasis is put on the supramolecular complexes located in the cytoplasmic membrane, namely, the flagellar motor and the rhodopsin complex.

Tatsuo Arai is a Professor at Graduate School of Engineering Science, Osaka University. He is also a project leader of Hyper Bio Assembler for 3D Cellular Innovation, supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology. He is specialize in robot mechanism analysis, design, and application. His research interest also covers micro manipulation system, limb mechanism robot, tele-operation, and bio-assembly.

Toshio Fukuda received the Doctor of Engineering from the University of Tokyo in 1977. He joined in the National Mechanical Engineering Laboratory, in Japan, from 1977. He joined in the Science University of Tokyo in Japan from 1982. He joined in the Department of Mechanical Engineering, Nagoya University in Japan as a professor from 1989. He joined in the Institute for Advanced Research, Nagoya University, Faculty of Science and Engineering, Meijo University in Japan, and Intelligent Robotics Institute, School of Mechatronic Engineering, Beijing Institute of Technology in China as a professor from 2013. He is mainly involved in the research field of intelligent robotic and mechatronic system, celler robotic system, micro and nano robotic system.