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Article

Role of bacterial flagella in bacterial adhesion of *Escherichia coli* to glass surface

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SUMMARY

Biofilms are communities of microbes dwelling on surfaces, which can pose potential hazards to human health and public hygiene due to their pathogenic properties. The development of biofilms is a dynamic process that begins with bacterial attachment to abiotic (non-living) surfaces. Therefore, it is crucial to understand the mechanism of bacterial adhesion to abiotic surfaces in the healthcare setting. Understanding the adhesion process also helps develop new methods to hinder biofouling in specific industrial settings and inhibit the growth of biofilm on medical devices that may cause infection. This study focused on flagella's involvement in adhesion, a key step in biofilm formation. We hypothesized that flagella, like pili, are involved in adhesion. In this study, we investigate the effects of flagella on bacterial adhesion to a glass surface by *Escherichia coli* (*E. coli*). In our assay, we reduced the number of flagella on *E. coli* cells through mechanical shearing and compared adhesion ability afterward. The evidence from our study suggests that the adhesion ability of *E. coli* cells increased with the shearing of flagella, indicating that bacterial flagella were disadvantageous for adhesion. We concluded that shearing caused increased attachment, as the decreased flagella may allow cells to attach more readily. The findings of the study contribute in several ways to our understanding of bacterial adherence to the glass surface and provide a basis for future researchers interested in developing novel agents that inhibit biofilm formation to different surfaces.

INTRODUCTION

Bacterial biofilms are microorganisms encased by extracellular polymeric substances (EPSs) attached to a foreign surface. The development of a biofilm is a dynamic process that often occurs in four stages. It is initiated by the bacterial attachment to a surface, followed by microcolony formation and biofilm maturation. The last stage of biofilm formation is dispersal (also termed detachment) and reversion to planktonic growth to colonize new areas (1). Current research makes it evident that biofilm adhesion is strongly associated with clinical biofilm-related infections and biofouling in industrial environments. Diseases triggered by biofilm buildup threaten many implanted or intravascular devices–such as mechanical heart valves, urinary catheters, pacemakers, endotracheal tubes, and joint prostheses–leading to extensive health issues in patients (2). Bacteria proliferate in biofilms, traveling on medical devices and infecting adjacent tissues, which can lead to fatal consequences. Cardiac and urinary devices are the most susceptible to infections compared to other medical devices. Left ventricular assist devices, heart valves, and cardiac implantable devices have an average incidence rate of 9.3% to 23.6%, causing bloodstream infection and a mortality rate over 25% (3). For urinary devices, 95% of urinary tract infections are catheter-related (4). Bacteria in the biofilm are also more resistant to antibiotics and disinfectants than bacteria without biofilm protection, making antimicrobial treatments ineffective. Once the biofilm matures, it becomes almost impossible to remove (4). In addition, biofilm formation and growth in drinking water distribution systems and water resources also create direct public health risks (5). The biofilm in the water distribution system becomes a breeding base for microbes and pathogens. Microorganism growth in biofilms will deteriorate the water quality and corrode the pipes. Pathogenic and opportunistic bacteria take advantage of these favorable conditions for growth. This distribution system further increases dissemination of the bacteria in the human population (5). According to the United States Environmental Protection Agency, biofilms act as a "slow-release mechanism" for persistent water resource contamination (5). Pollutants are constantly released from the biofilm, making it a direct threat to public health and hygiene (5).

Bacterial attachment is often a dynamic process that relies on specific adhesive organelles and substances (pili, flagella, extracellular polymeric substances) or nonspecific interactions with the surface (covalent bonds, Van der Waals forces, electrostatic forces) (1). Flagella are of particular interest to this study. Flagella and pili are filamentous polymeric protein structures attached to the surfaces of bacteria––two important organelles present in most bacteria. Previous study has established pili's indispensable role in adhesion (6). Traditionally, flagella are regarded as a motility organelle that propels the bacteria and enables chemotaxis (6). However, the structural similarities possessed by pili and flagella could indicate that flagella have a similar role in adhesion like pili. We made several speculations about the potential role of bacterial flagella in adhesion based upon their unique structure. Unlike pili, which are short and rich in number, flagella are long, thin, and whip-like appendages, and they are loosely distributed around the surface. Flagella, nearly ten times longer than the bacterial body, can interact directly with the objects' surfaces over a long distance (7). It is also reasonable to speculate that the flagella may help overcome surface repulsive forces and facilitate cell diffusion along the surface by the colossal force generated by the rotation of the flagellar motor. The flagellar motor for the

prokaryotic flagellum can reach 100,000 rpm for a particular species, a rate that exceeds the rotation of jet engines (7).

Recently, several studies showed intriguing results in flagella's involvement in adhesion. One study discovered that flagella function as adhesin organelles and help to subdue unideal surface topography (8). This case has shown that *E. coli's* flagella are capable of reaching microscale crevices and aid adhesion by exploiting the extra surface area brought by those hollows. Another study reported the whole bacteria flagella as a significant adhesin to host cells for pathogens, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Clostridium difficile* (9). This case study confirms the importance of flagella in aiding adhesion indirectly through mediated adhesion to eukaryotic cells via motility and directly by sticking to the targets (9). Despite some exciting discoveries, the existing knowledge on flagella-mediated adhesion is limited. Thus, many aspects of flagella's role in adhesion requires further investigation.

In this study, we asked whether the removal of bacterial flagella affects adhesion to abiotic surfaces. We hypothesized that the removal of bacteria flagella would decrease the adhesion ability. To test this hypothesis, we removed flagella from a strain of *E. coli* by mechanical shearing and tested its adhesion ability to a glass surface. We presumed the increased shearing caused fewer numbers of flagella. We discovered that the increased shearing corresponded with the increase in bacterial adhesion. The result suggests that bacterial flagella are disadvantageous for bacterial adhesion to the glass surface. Our study provided direct evidence to illustrate the role of bacterial flagella in attachment to a specific media. It also provided future researchers with a procedure to evaluate the effect of the shearing of bacterial flagella in adhesion to different surface media.

RESULTS

We selected PQE-EGFP *E. coli* cells as the experimental group and PQE-mCherry *E. coli* cells as our control (**Figure 1**). We chose green and red fluorescent proteins specifically because their contrast helps in data collection and visualization. The control and experimental groups were grown in identical conditions, except the PQE-EGFP-expressing *E. coli* cells were mechanically sheared through the passage of a syringe-needle system. We decided to extract the flagella of PQE-EGFP cells mechanically because chemical methods could change other bacterial properties that could affect adhesion. The PQE-EGFP-expressing cells' flagella were removed through the passage of the syringe system for 5, 10, 20, and 40 passages before the experiment.

In the experiment, we transferred the same quantity of cells from the experimental and control groups measured by optical density (OD) test on the glass slides. We used a fluorescence microscope to collect the images of different sections of the glass slides (**Figure 2**). We calculated the experimental group's adhesion ability as a ratio of the number of PQE-EGFP-expressing *E. coli* cells to PQE-mCherry-expressing E. *coli* cells. By calculating the adhesion ability as a ratio, we effectively ruled out the possibility of different surface topography of glass slides that could affect adhesion. Since the experimental and control groups used different plasmids, we included an experimental point in which neither of the cells underwent the shearing procedure. We found that the relative adhesion ability for PQE-EGFP cells is 0.40 ± 0.14, which means the adhesion ability of PQE-EGFP-expressing *E. coli* is 40% of PQE-mCherry-expressing *E. coli* cells without shearing. Thus, when the PQE-mCherry cells' adhesion remained consistent in all experiments, an increase of the adhesion ratio would indicate that the cell adhesion ability of the PQE-EGFP cells increased or vice versa.

We calculated the adhesion ratios with varying number of shearing passages of PQE-EGFP-expressing cells through the syringe-needle system. The relative adhesion ratio remained around 0.50 for 5, 10, and 20 passages through the syringe system. However, when the passages increased to 40, there was a sharp increase in adhesion ratio to 0.60. As we increased the number of the passages through the syringes-needle system, the number of flagella was proposed to decrease gradually, and the bacteria's adhesion ability was observed to increase sharply on the interval of 20 to 40 passages (**Figure 3**). The result demonstrated a steep rise in the adhesion ability of PQE-EGFP-expressing cells when the cells surpassed the 20-to-40 passages point. We concluded that shearing caused increased attachment, suggesting the decreased flagella may allow cells to attach more readily.

DISCUSSION

In this experiment, we investigated the effects of flagella on *E. coli's* adhesion ability. We can draw the following conclusion: *E. coli's* adhesion ability to glass surface increases with increased cell shearing passages, indicating that the reduced quantity of flagella may aid cells to attach more readily. The experiment result indicates that our initial hypothesis is not true. Interestingly, bacterial flagella seem not

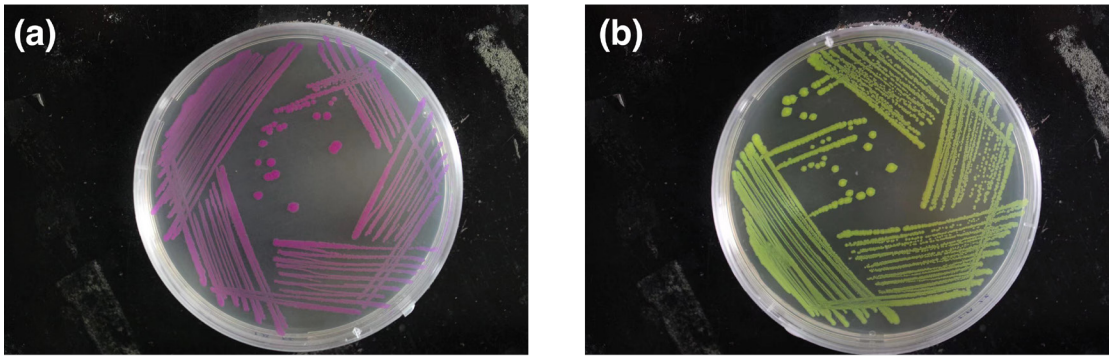

Figure 1: PQE-mCherry-*E. coli* cells and PQE-EGFP-*E. coli* cells. (a) PQE-mCherry-*E. coli* cells (reference group) and (b) PQE-EGFP-*E. coli* cells (experimental group) grown on LB medium at 37° C.

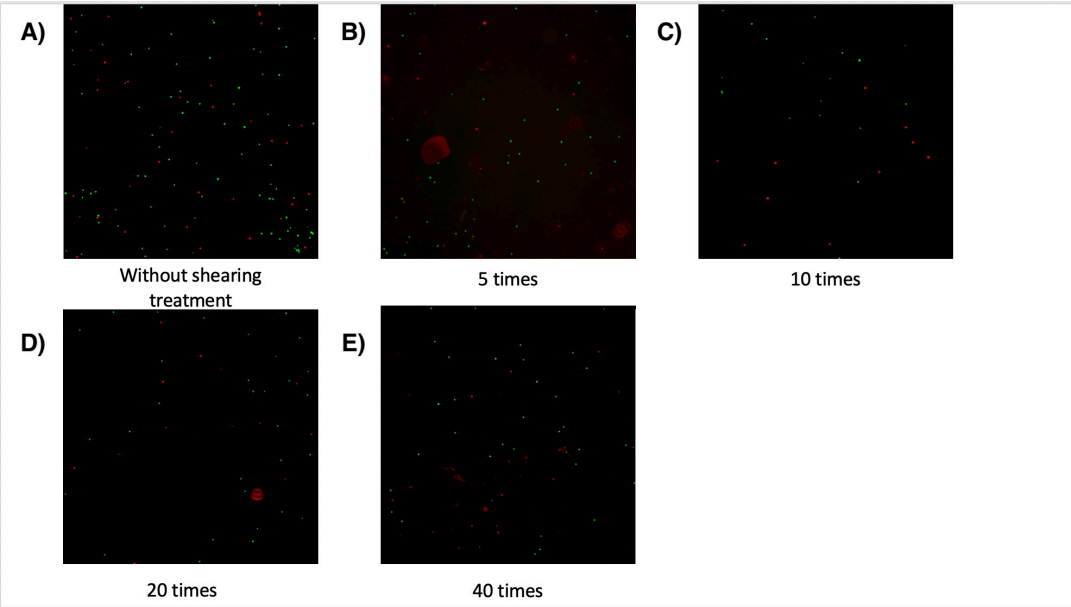

Figure 2: *E. coli* cells adhesion with increasing passage through syringe system. Representative fluorescent images illustrating 0 passages (A), 5 passages (B), 10 passages (C), 20 passages (D), and 40 passages (E) of PQE-EGFP-expressing cells through the syringe-needle system (n=5). The green dots represented PQE-EGFP-expressing cells and the red dots represented PQE-mCherry-expressing cells. The image was taken with a fluorescence microscope (Nikon-Eclipse-Ti) in the most densely populated area.

to be advantageous for bacterial attachment in our assay.

There are several possible explanations for the pattern we discovered. In our assay, the bacteria cells we cultured were considered in a free-swimming state. The transition from the planktonic lifestyle to the sessile (free floating) lifestyle should occur, which could affect the role of flagella in the attachment. The properties of glass slides should be taken into consideration as well. Studies showed that type I fimbriae and flagella strengthened the adhesion to the hydrophobic surface but reduced adhesion to hydrophilic surfaces (10, 11). Since glass is a hydrophilic material, the increased adhesion could be caused by the flagella reduction. One possibility is that the flagella that originally obstruct adhesion are removed, resulting in an increase in adhesion.

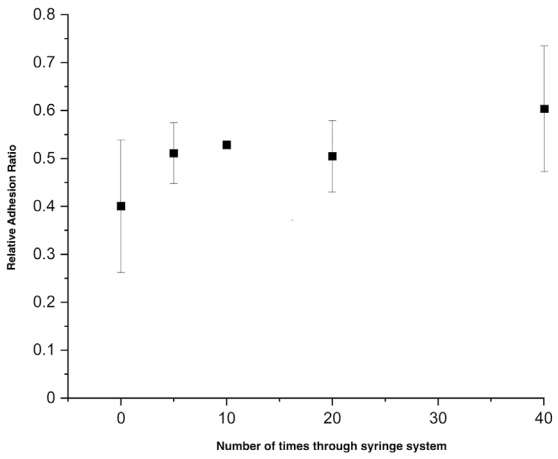

Figure 3: *E. coli* relative adhesion ratio. The vertical axis indicates the relative adhesion ability, which is calculated by the ratio of PQE-EGFP-*E. coli* to PQE-mCherry-*E. coli* (n = 5, mean, and max and min SD). Error bars present the range. The horizontal axis is the number of passages that cells pass through the syringe system. The 0 passage point is the experimental point where neither PQE-EGFP *E. coli* nor PQE-mCherry *E. coli* underwent the shearing procedure.

The observed increase in adhesion ability as the shearing of flagella could be attributed to cyclic di-GMP, a second messenger for signal transduction in diverse bacterial species that controls various cellular processes such as biofilm formation and surface adaptation. Cyclic di-GMP's most distinct role is its ability to regulate the decision of switching from a planktonic lifestyle to a sessile lifestyle when it is advantageous to slow down. Recent studies discovered that cyclic di-GMP activates the flagella brake protein YcgR. This protein reduces swimming speed by binding to the flagellar motor when it is more advantageous to slow down (12). For example, the braking action occurs when bacteria enter starvation or stationary growth condition (12). In our study, cells were sheared mechanically. The loss of flagella due to shearing implies a decrease in cell motility. We theorize that significant loss of motility may be another disadvantageous circumstance like starvation, when remaining as free-swimming bacteria is no longer beneficial. Thus, the cyclic di-GMP level increases and triggers YcgR to slow the flagella motor embedded in the cell envelope and start surface attachment. However, since more flagellar filaments are removed with the increasing shearing, the bacteria may reach a point where it loses all the flagellar filaments. Hence losing their motility, the bacteria initiate surface attachment and stationary growth without the YcgR binding to the flagellar motor. However, the assumption will require future studies to provide additional evidence.

We speculate that significant loss of flagella that leads to considerable loss of motility could be another scenario when the cyclic di-GMP level increased, resulting in increased adhesion. This may be a plausible explanation for the abrupt increase in adhesion ability between the 20 to 40 passages in our data. We speculate that the PQE-EGFP cells lost most of

the flagella and experienced a substantial decrease in motility after being sheared 40 passages. Hence, we see an abrupt increase in adhesion ability when the loss of motility exceeds a certain threshold. However, this speculation needs the support of supplementary experimental data. Future studies should quantify the number of flagella and measure cell motility after shearing.

Considering that the subtle differences in surface characteristics of different glasses can lead to bacterial adhesion differences, we used PQE-mCherry as a control to study the effect of flagella on adhesion. We observed that the adhesion of PQE-EGFP bacteria was 40% of that of PQE-mCherry bacteria, which may be caused by the different adhesion abilities of bacteria in different growth states. To further confirm the reliability of our experiment, future studies may consider exchanging the control group and the experimental group for a repeated experiment, which means using PQE-mCherry as experimental group and PQE-EGFP as control group. However, since PQE-mCherry cells were used as a control in all experiments, we believe that the difference in bacterial adhesion ability between PQE-mCherry cells and PQE-EGFP cells did not affect the pattern caused by the number of flagella.

Future studies could replicate our experiments on various surface media with several improvements and new study focuses. First, future researchers should prioritize studying materials commonly used in water lines, intravascular devices, and built-in devices. As mentioned in the previous paragraph, quantification of flagella is required to explain the abrupt increase in adhesion in the 20-to-40 passages interval. Our experiment is based on an intuitive assumption that the increasing passages of shearing extract an increasing number of flagella. However, this assumption needs to be experimentally validated. In addition, the idea of a motility threshold where bacteria lose a considerable number of flagella and motility that induce an abrupt adhesion ability boost can be investigated thoroughly. The relationship between adhesion ability and cell motility can be an interesting start. A motility assay can be added to our experiment to show an explicit association of cells' motility and adhesion ability.

Strategies to enhance the study might involve using a growth or viability assay like the tetrazolium reduction (MTT) assay to indicate the number of cells instead of an OD measurement. OD measurement cannot distinguish live cells from dead cells. An identical OD measurement could mean the same concentration of live and dead cells altogether. Nevertheless, if the shearing caused more dead cells in the experimental group, it may influence the result. Hence, a viability assay will enhance the study's strength. Another alternative to our study is to use flagella-less bacteria. The benefit of using flagella-less bacteria strains is to guarantee cells' viability as they do not need to undergo any mechanical shearing procedure. Lastly, although we think a consistent control strain is sufficient for assuring the study's accuracy, researchers could make alternatives on the choice of the control strain. A control strain that behaves identically with the experimental strain would be the most ideal.

MATERIALS AND METHODS

Our study used *E. coli* K-12 MG1655 cells harboring the plasmid (PQE-EGFP) to express green fluorescence and *E. coli* cells harboring plasmid (PQE-mCherry) to express red fluorescence. We calculated the adhesion ability as a ratio of the total number of PQE-EGFP *E. coli* cells to the total number of PQE-mCherry *E. coli* cells attached in order to eliminate confounding variables such as the difference in glass surface topography.

Preparation of *E. coli* cells

We used a standard transformation protocol to introduce plasmid DNA (PQE-EGFP and PQE-mCherry) into competent *E. coli* K-12 MG1655 cells. We selected a single fresh colony of the desired strain from an agar plate and inoculated it into a liquid medium. In the mid-log growth phase, we harvested the cells. The prepared cells mixed with the DNA plasmids using a ratio of 50μL of the competent cells to 5ng of plasmid DNA. Then, we heat-shocked the competent cells in a water bath set to 42°C for 90 seconds and incubated them in ice for 2 minutes afterward. In the recovery step, we transferred cells to 250 μL of Luria-Bertani broth (10 g NaCl, 5 g yeast extract, 10 g tryptone per liter) to each tube and mixed by gently flicking the tubes. The *E. coli* cells were incubated again in a 37°C water bath for 30 minutes. Later, the cells were plated to LB agar plates (10 g NaCl, 5 g yeast extract, 10 g tryptone per liter, 15 g agar per liter) containing requisite antibiotics including 100 μg/mL ampicillin and grown to maturation at 37°C in a petri dish. We selected single colonies that expressed the correct fluorescence proteins and transferred them to LB broth with appropriate antibiotics (Ampicillin 100 μg/mL). The cells were grown in a rotatory incubator for 24 hours and collected by centrifugation for later flagella extraction.

Bacterial flagella extraction method

We referenced Berg and Tedsco's protocol to remove flagella of *E. coli* PQE-EGFP cells (experimental group), not those of *E. coli* PQE-mCherry cells (control group), by the passage of the suspension varying from 5 to 40 passages between two syringes equipped with 26-gauge needles connected with an 8 cm length of polyethylene tubing (0.58 mm internal diameter) (13). PQE-EGFP cells were split into four experimental groups sheared 5, 10, 20, and 40 passages.

Experimental procedure

There was a point prior to the experiment when neither of the cells underwent shearing procedure. In our experiment, approximately the same amount of PQE-EGFP-*E. Coli* and PQE-mCherry-*E. coli* cells had been added to each slide and incubated for 10 minutes. The concentration of both groups was examined by measuring the OD at 600 nm, ensuring the experimental and control groups both had the exact OD600 readout of 1.0. Then, *E. coli* cells were dripped on the glass slides using a 10 μl volume pipettor. The slides were washed two times by distilled water after the incubation to remove unattached or dead cells. We then observed each slide using a fluorescence microscope (Nikon-Eclipse-Ti). We looked for clusters of successfully attached bacteria manually and took images of the glass broken down into multiple sections. We counted manually and calculated the ratio of the experimental groups' number of *E. coli* to the control group's number to assess the bacteria adhesion ability afterward.

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