Electrophoretic Mobility of *Bacillus subtilis* Knockout Mutants with and without Flagella

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Mutants of *Bacillus subtilis* 168 strain were obtained by inactivation of a specific gene by homologous recombination with the plasmid pMutinT3. The cell surface properties of these strains were characterized by measuring the electrophoretic mobility of the cells as a function of pH and ionic strength. The surface properties were different for the strains possessing flagella on their cells and strain FlgB, having no flagellum, due to knockout of the corresponding gene. The cell surface properties of the strains possessing flagella become similar to those of strain FlgB after acid treatment. It was confirmed that the acid treatment degraded the flagella without causing any apparent structural change on the cell surface via observations made using atomic force microscopy, transmission electron microscopy, and scanning electron microscopy. These results indicate that the flagella are a key factor influencing cell surface properties.

Many microbes exist attached to various surfaces in natural environments. The attachment of microbial cells is greatly influenced by cell surface properties, and the electric charge on a microbial cell surface has been a key feature for characterizing cell surface properties. To estimate the electric charge, the velocity of movement of the cells suspended in a buffer under an electric field has been measured. The electrophoretic mobility (EPM) of the cells, obtained by dividing the velocity by the strength of the electrical field, has been used to estimate the electric charge on the cell surface.

Recently, strange electrokinetic behavior of some colloidal particles, including bacterial cells, has been noticed, in which the colloidal particles can be mobilized in an electric field even when the ionic strength of the buffer becomes quite high (2, 8, 12, 18, 19). Conventional thought is that colloidal particles cease to move under conditions of high ionic strength due to depression of the thickness of the electric double layer by counter ions. This discrepancy between experimental results and the conventional theory demands reconsideration of the EPM value and the cell surface properties estimated from EPM data.

Ohshima (15, 16) has proposed a new theory that can explain the strange behavior of some colloidal particles. According to this theory, the electrical charge in a polymer layer existing at the particle surface is the driving force that mobilizes the colloidal particles under conditions of high ionic strength. The depression of the electrical double layer at the surface (10, 12). Hence, the soft layer at the cell surface plays an important role in bacterial attachment, and that softness has been analyzed (2, 9, 12, 19). Recently the cell surface softness of fibrillated and nonfibrillated streptococcal cell surfaces has been directly measured with an atomic force microscope (AFM) (21, 22).

To reveal the factors influencing the polymer layer, mutant strains with characteristic cell surface properties seem to be quite useful. However, it has generally been difficult to obtain a strain in which only a gene affecting the cell surface properties is mutated while other properties are kept unchanged. If we can produce mutants by regulating specific genes, we can have a set of mutants to use to sort and categorize the factors affecting the cell surface properties. Recent progress in molecular biology makes this possible. The genome sequence of *Bacillus subtilis* was completely determined in 1997 (11), followed by transcriptome or proteome studies. An effective way to analyze functions of the genes in studies concerning *B. subtilis* is to produce knockout mutants (14). It is expected that by inactivating the genes related to cell surface properties, we can obtain the ideal set of mutants.

In the present study, we investigated the cell surface properties of the strains of *B. subtilis* obtained by inactivating genes corresponding to the formation of flagella. We confirmed that the cell surface properties are greatly affected by flagella.

**MATERIALS AND METHODS**

Knockout mutants. In this study, 20 strains of *B. subtilis* knockout mutants of genes and the wild-type strain 168 were used. The mutants were obtained by

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homologous recombination. To prepare the insert DNA necessary to obtain the mutant, a partial region upstream of the target gene was amplified by PCR. This insert DNA was integrated into the plasmid pMutinT3, with the erythromycin and ampicillin resistance genes included as selective markers (13, 20). Escherichia coli C600 was transformed with this plasmid and cultured in Luria-Bertani (LB) medium with ampicillin. A sufficient amount of the plasmid was obtained from the cultured cells with the PE Applied Biosystems miniprep kit. B. subtilis 168 (wild type) was subsequently transformed with the plasmid. The FlgB, MotA, and YhcN strains had the flgB, motA, and yhcN genes, respectively, knocked out. The phenotypic differences of these strains are that they have no flagella, no motility, and no agellum, no motility, and no agellum, respectively.

The FlgB, MotA, and YhcN knockout mutants and the wild-type strain 168 were the main strains used in this study. The FlgB, MotA, and YhcN strains had the flgB, motA, and yhcN genes, respectively, knocked out. The phenotypic differences of these strains are that they have no flagellum, no motility, and no pilus, respectively.

These strains were cultured in LB medium containing Lennox LB broth (20 g liter⁻¹) (Difco); erythromycin was added to the medium at 0.5 mg liter⁻¹ for the knockout mutants. Cells in stationary phase, cultured in the LB medium for about 20 h at 27°C with shaking at 100 rpm aerobically, were centrifuged at 8,000 × g for 10 min at 4°C. After being washed twice with 10 mM NaCl solution or 0.154 M phosphate buffer, a sucrose solution (0.5 M), and distilled water. To measure the EPM at various pHs, the cell suspension in the 10 mM NaCl solution was mixed (1:19, vol/vol) with either a modified phosphate buffer solution (pH 2) or the phosphate buffer solution (pH 7.4) described above. The cell suspension was used for the EPM measurement as a function of pH or ionic strength.

AFM. Bacterial cells were cultured until the early stationary phase, and the cells were centrifuged at 8,000 × g for 10 min at 4°C. After being washed twice with distilled water, the cells were resuspended in distilled water. A droplet of the cell suspension was carefully placed on a cover glass, which was cleaned by sonication beforehand. After desiccation to fix the cells on the glass, they underwent preliminary scanning several times and were observed with an SPM9500J3 scanning probe microscope (Shimadzu). Contact-mode images were taken with an applied force maintained below 4 nN at a scan rate of 0.5 Hz. We used OMCL-TR800PSA-1 Si₃N₄ cantilevers (Olympus) with a length of 100 or 200 μm and a spring constant of 0.57 or 0.15 N/m, respectively, to measure both the height and deflection of the specimen. We used NCHR-20 cantilevers (Tomo) with a length of 126 μm, a spring constant of 37 N/m, and a resonance frequency of 332 Hz in dynamic-mode operation to obtain the high-resolution images. The AFM was operated at −0.194 V, and the dynamic-mode images were taken at a scan rate of 1.5 Hz to obtain both the height and deflection of the specimen.

**RESULTS**

Cell EPM as a function of ionic strength. We measured the EPMs of B. subtilis cells at various ionic strengths. The EPM of the cells of the four strains changed along with the ionic strength as shown in Fig. 1A. The FlgB strains showed slightly lower EPMs, with negative values, than the other strains, especially at lower ionic strengths. Regardless of the difference, the negative EPM of each strain approached a nonzero value,
which is characteristic of colloidal particles carrying the polymer layer at their surfaces (15, 16). According to Ohshima (15, 16), the EPM of these particles can be expressed by the equation

\[
\mu = \left( \frac{\varepsilon_r}{\varepsilon_0} \right) \left( \frac{\psi_0/\kappa_m + \psi_{DON}/\lambda}{1/\kappa_m + 1/\lambda} \right) + \frac{zeN}{\eta^2} \quad (1)
\]

where \( \mu \) is the electrophoretic mobility of the particles, \( \varepsilon_r \) is the relative permittivity of the medium in which the particles are suspended, \( \varepsilon_0 \) is the permittivity of a vacuum, \( \eta \) is the viscosity of the medium, \( \psi_{DON} \) is the Donnan potential of the polymer layer, and \( \psi_0 \) is the potential at the boundary between the layer composed of polymers at the particle surface and the surrounding solution. In addition, \( \kappa_m \) can be interpreted as the Debye-Hückel parameter of the polymer layer, \( \lambda \) is a parameter whose reciprocal has the dimension of length and which corresponding to the softness of the polymer layer, \( z \) is the valence of charged groups in the polymer, \( e \) is the electric unit charge, and \( N \) is the density of the charged groups in the polymer layer.

Ohshima’s theory can explain the EPM change as a nonzero value is approached at higher ionic strengths by considering the presence of a charged polymer layer at the cell surface. The changing EPM pattern with the ionic strength in Fig. 1A indicates the presence of a polymer layer at the cell surfaces of the four strains. We can characterize the polymer layer by determining the density of its charged groups \( (N) \) and softness \((1/\lambda)\) by fitting the experimental results to the theoretical curve from

![Figure 2](http://jb.asm.org/)
equation 1, as shown in Fig. 2A and C. The three strains other than the FlgB strain showed similar values. For the FlgB strain, however, the density of the charged groups was about half of that of the other strains, while the softness was about 1.5 times greater than that of the others.

**Cell EPM as a function of ionic strength after acid treatment.** We measured the EPMs of the cells at various ionic strengths after acid treatment (Fig. 1B). After the acid treatment, the EPMs of the wild-type, MotA, and YhcN strains became similar to that of the FlgB strain without acid treatment. Interestingly, the EPM of the FlgB strain was not significantly affected by the acid treatment.

The softness and density of the charged groups of the polymer layer after acid treatment are shown in Fig. 2B and D. All of the strains showed almost the same values. Figure 2 shows that the values for the wild-type, MotA, and YhcN strains were different from those for the FlgB strain before the acid treatment, whereas after the acid treatment these values became similar to those for the FlgB strain.

**Cell EPM as a function of pH.** The EPM of *B. subtilis* cells was measured at various pH values (Fig. 3A). The negative EPM of the strains other than the FlgB strain showed a peak at pH 4. On the other hand, the EPM of the FlgB strain remained almost constant from pH 5 to 9 and did not show such a peak. The EPM of microbial cells seems to reflect the electric charges due to ionization of various functional groups on the cell surface. The ionization of these functional groups changes as a function of pH monotonically; however, the measured EPM for the wild-type, MotA, and YhcN strains changed in a skewed manner between pH 3 and 4. This unusual pattern (Fig. 3A) cannot be explained by simply considering the extent of ionization of functional groups, as described above.

**Cell EPM as a function of pH after acid treatment.** Consider the case of two strains of bacterial cells whose changes in EPM as a function of pH are totally different from each other. If the EPM values of one strain at pH 2 and 3 and the values of another strain at pH 4 to 9 are plotted together, we may obtain a relationship between EPM and pH showing a gap at the point where they connect, as seen in Fig. 3A. Thus, the cells of the three strains (wild type, MotA, and YhcN) may behave as if they were different strains below pH 3 and above pH 4, leading to the consideration that cell surface properties might be altered between pH 3 and 4.

To test this hypothesis, we treated the cells with an acid solution of pH 2 and then measured the EPM value (Fig. 3B). Figure 3B shows that the EPM value at pH 4 became more negative than that seen in Fig. 3A, and the peak at pH 4 disappeared. The negative EPM between pH 5 and 9 was almost constant and actually became smaller than that of the intact cells (Fig. 3A). It is noteworthy that the changing EPM pattern after the acid treatment is similar to that of the FlgB strain before the acid treatment, and this result can be reasonably explained by assuming the disappearance of the flagella after the acid treatment of the wild-type, MotA, and YhcN strains. This assumption is strongly supported by the finding that the flagella of *B. subtilis* degraded into the monomer flagellin in an acid solution of pH 3.2 (5).

**Observations by AFM.** In the FlgB strain the gene relating to the formation of flagella is inactivated. Thus, we expect to find no flagella on cells of this strain. To confirm this, cells of both the wild-type and FlgB strains before and after acid treatment were observed by AFM. From the contact-mode images obtained by AFM (Fig. 4A and B), it was confirmed that the FlgB strain originally had no flagella and that in the wild type the
FIG. 4. AFM gray-scale height images obtained in the contact mode (A and B) and deflection images obtained in the dynamic mode (C) for the cells of the *B. subtilis* wild-type (A and C) and FlgB (B) strains. Panels 1, intact cells; panels 2, cells treated with an acid solution at pH 2.
flagella disappeared after the acid treatment. In addition, AFM images of the MotA and YhcN strains clearly showed that the cells lost their flagella after the acid treatment (data not shown).

Under acidic conditions (pH 2), cell surface components other than flagella may be affected. The dynamic-mode topographic images of the wild-type cells showed that this is not the case (Fig. 4C). The dynamic-mode images of the FlgB, MotA, and YhcN strains also showed no additional change other than the disappearance of flagella due to the acid treatment. Analysis with transmission and scanning electron microscopy also confirmed these results (data not shown).

**DISCUSSION**

In this study, we measured the EPMs of various strains of *B. subtilis*. From the fact that EPM values approached nonzero values at higher ionic strengths (Fig. 1A), it was confirmed that all of the cells of these strains have a polymer layer at their surface. Among the strains, the FlgB strain is significantly different from the other strains in that the softness (1/N) of its polymer layer was greatest, while the density of its charged groups (N) was lower than those of the other strains (Fig. 2A and C). These characteristics seem to be caused by the knock-out of the flgB gene. When the other three strains were treated with an acid solution, the dependence of the EPM on ionic strength was similar across the board (Fig. 1B), with little difference from that of the FlgB strain. Consequently, these three acid-treated strains showed similar N and 1/N values (Fig. 2B and D), and these values were close to those of the intact FlgB strain. These results indicate that a similar component, probably flagella, at the cell surfaces of these three strains (wild type, MotA, and YhcN) was altered by the acid treatment, causing the cells to resemble those of the FlgB strain.

The EPMs at various pH values for the wild-type, MotA, and YhcN *B. subtilis* strains showed a peak at pH 4 (Fig. 3A), whereas after the cells were treated with the acid solution at pH 2, the peak disappeared and the negative EPM value at pH 5 to 9 decreased (Fig. 3B). In contrast to the case for these three strains, the EPM of the FlgB strain did not show any such large changes resulting from the acid treatment. From these results it was deduced that the acid treatment caused the decomposition of flagella into flagellin, which resulted in an EPM change along with the changing pH of the cells that was similar to that of the FlgB strain, which had no flagella to begin with.

The bacterial flagellum is composed of the hook, basal body, and filament. The filament consists of flagellin, the major filament protein (1, 4, 6). The flagella of *B. subtilis* are disaggregated into flagellin monomer at pH 3.2 (5). In the present study, it was confirmed by using AFM that the peritrichous flagella disappeared completely due to the acid treatment at pH 2 (Fig. 4). These observations are consistent with the significant change in EPM values between pH 3 and 4 for the wild-type, MotA and YhcN strains; if the flagella had only partially disappeared, then the EPM change would be smaller.

Moreover, in the observations made with the dynamic-mode AFM topographic images (Fig. 4C) and both the transmission and scanning electron microscope images (data not shown), no change other than the disappearance of flagella could be found at the cell surfaces of these strains. Therefore, it was considered that only the flagella were decomposed during EPM measurement at pH values lower than 3, and there were no apparent signs of any other surface changes to the cells. It is also noteworthy that the flagellin, containing 304 amino acid residues, is richer in acidic than in basic residues (40 and 33 residues, respectively) (3). This may cause the less negative EPM at pH 4 to 9 after the acid treatment.

Although further analysis, such as chemical composition analysis of the outermost layer (2 to 5 nm thick) of the cell surface (7) with X-ray photoelectron spectroscopy, may prove other effects of the acid treatment on the cell surface, at present we can divide the actual EPM data into two sets: the data for pH values higher than 4 (cells still possessing flagella) and the data for pH values lower than 3 (cells lacking flagella). The actual data for the EPM as a function of pH should consist of the two data sets, as schematically illustrated in Fig. 5. The actual data from pH 5 to 9 should correspond to the cells possessing flagella, and the data at pH 2 and 3 should correspond to the cells lacking flagella.

In the present study, we found that the flagella play a very important role in characterizing certain cell surface properties, such as softness and density of the charged groups in the polymer layer. The disappearance of the flagella as a result of the acid treatment affected the cell surface properties, as indicated by the change of EPM as a function of pH or ionic strength. In addition to the four strains used in the present study, we performed the same experiments with 17 other...
strains (having flagella but with knockout genes relating to cell surface structures such as the pilus, capsule, cell wall, and adhesin and to sporulation) and obtained almost identical results (data not shown). Therefore, the effect of flagella on cell surface properties seems to be similar among different strains of *B. subtilis*.

The strategy adopted in the present study is promising, because we can obtain a set of mutants with the intended surface properties by inactivating a target gene, although the alteration of the surface polymers at the molecular level which is important in specific interactions may be difficult to detect. We can then determine the factors influencing those surface properties. Further investigation will involve the relationship between cell surface properties and cell attachment.

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