Surface Characteristics of Bacterial Cells Isolated from River Sand Grains and Their Relevance to Attachment

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Bacteria on the surface of river sand grains were successively detached by changing the number and strength of washing procedures and then divided into different fractions. The ratio of slow-growing organisms in each fraction increased as the number of washings increased and intensified by sonication. The cells of these slow-growing strains were more hydrophobic, less negatively charged and had greater isoelectric point (IEP) values compared with fast-growing strains. The strains having greater IEP values attached more easily to a glass and a plastic surface than the strains having smaller IEP values. These findings lead to an assumption that the slow-growing bacteria have surface characteristics enabling the cells to attach more firmly to substrate surfaces.

Key words: Sand bacteria, Attachment strength, Growth rate, Cell surface, Isoelectric point

Introduction

The microbial cell surface is the outermost fringe where the interactions between the microbe and its environment take place. Thus, the surface characteristics of microbial cells play an important role in the behavior of microbes in the environment. A relationship has been found between the growth rate and surface characteristics of bacteria isolated from grassland and paddy soil: slower-growing isolates have less electric charge8,12 and are more hydrophobic6 at their cell surfaces.

The surface characteristics appear to affect the microbial behavior through cell attachment to, or detachment from, various surfaces in the environment. However, there have been few studies concerning the relationship between the surface characteristics of microbes and their attachment state as it is difficult to observe these relationships if a solid sample having a rough surface and complex structure is used.

As the surface of sand grains is expected to be relatively smooth and poor in porosity, it may serve as an appropriate site to examine the relationship between the surface characteristics of microbial cells and their attachment state. In this study, therefore, we isolated bacteria from the surface of river sand grains by changing the strength of the washing used to detach the cells; i.e. a stronger procedure was needed to detach the bacteria which were attached more firmly. We examined three groups of the isolates differing in their attachment strength and found a relationship between the surface characteristics, the attachment strength and the growth rate of these isolates.

Materials and Methods

River Sand

At the riverside of an upstream region of the Hozu
river (Kyoto, Japan) about 400m to the northeast of Hozu station (Japan Railway), coarse sand grains (water content 7.91%, pH 7.6) were sampled (June 8, 1993) in a sterilized bottle and brought back to our laboratory within a few hours and kept in a refrigerator (4°C) for two days before isolating bacteria from their surfaces.

**Washing out of bacteria from the sand grains**

About 1g of the sand grains was put into a bottle containing 100 ml of sterilized water and shaken by hand up and down 20 times. Five milliliters of the water was taken out as a sample to incubate as described below and the rest of the supernatant was discarded. We counted this procedure as washing time #1. Another 100 ml of sterilized water was added again to the bottle and the sand sample was washed as described above. This washing procedure was repeated successively. After being washed 10 times, the sand sample was sonicated for 2 min at 40 kHz and 20 W (UA50D, Shinmeidai Co., Japan) in 100 ml of sterilized water.

**Isolation of sand bacteria from each bacterial fraction based on colony formation curve**

Each of the five milliliter samples, obtained by washing the sample of sand grains two and ten times and by sonicating, were diluted with sterilized water and plated on a nutrient broth (NB) agar medium or a 100-fold dilution of nutrient broth (DNB) agar medium containing 0.1 g of meat extract, 0.1 g of peptone, 0.05 g of NaCl and 10 g of agar in 1 liter of water (pH 7.0). Four replicates for the NB plates and twenty replicates for the DNB plates were used. The plates were incubated at 25°C for 2 weeks and the number of colonies was counted everyday. Colonies appearing on different days were marked with different colors to distinguish them. From the plates using DNB agar, strains were isolated as follows. At the end of the incubation period, more than 100 strains were isolated at random from each of the 3 samples. Each of these isolates was maintained in DNB with 0.4 % agar as a stab culture. From these strains, about 30 strains were chosen so as to contain about 10 strains from each of the three groups differing in colony appearance time as 0–3, 4–7 and 8–13 days and used for further experiments.

**Culture and preparation of bacterial sample**

The isolates were cultured in the DNB medium shaken aerobically (100 rpm) at 27°C until an early stationary phase was reached. The cells were centrifuged (13,000 × g, 4°C, 10 min) and washed twice with 10 mM NaCl solution or 10 mM phosphate buffer saline (PBS, containing 0.142 g of Na₂HPO₄ and 0.526 g of NaCl in 1 liter of water; pH 7.0), and the cells were resuspended in the washing medium.

**Electrophoretic mobility (EPM) of bacterial cells**

The stationary phase cells suspended in the 10 mM NaCl solution were mixed with 10 mM of PBS (pH was adjusted from 2 to 9 with 20 mM HCl or NaOH aq. solution) at the volume ratio of 1 to 20. The EPM of the cells was measured at 25°C with a PEN KEM system 3000 (USA).

**Hydrophobic interaction chromatography (HIC)**

The hydrophobicity of the isolates was examined by following the procedure of Smyth et al.13) and Sunairi et al.14). Sixty µl of the stationary phase cells suspended in 10 mM PBS (pH 7.0) was applied to the top of a Pasteur pipette filled with 0.9 ml of octyl-Sepharose CL-4B (Pharmacia Biotec); the suspension was adjusted beforehand to read O.D.₆₆₀=0.4–0.6 when diluted with 3 ml of the cell-free PBS. After 5 min, the cells were eluted with 3 ml of the PBS. The percentage of retained cells was calculated by the following equation,

$$\text{percent retention} = \left(1 - \frac{A}{A_0}\right) \times 100$$

where A is the turbidity of the eluted suspension and A₀ is the turbidity obtained when the initially applied 60 µl of cell suspension was diluted with 3 ml of the cell-free PBS.

**Cell attachment**

The number of cells attached to a glass and a plastic surface was examined by following the procedure by Morisaki9). The stationary phase cells suspended in 10 mM NaCl solution were diluted with 10 mM PBS (pH 7.0) to a concentration of 1 × 10⁷ cells/ml. A por-
tion of the cell suspension, 0.4 ml, was put into a well of a glass and plastic plate (Lab-Tek Chamber Slide, Nunc, USA). After 5 hours, the cell suspension was removed gently and the plate surface was rinsed 3 times with 0.4 ml of the PBS and then dried in air. The number of cells attached to the plate surface was counted with a differential interference microscope (BX50, Olympus, Japan). The number of cells in one field of view (250 × 350 μm²) was counted at five different positions and averaged.

Results

Colony forming curve of sand bacteria

The fractionated bacterial samples were diluted and plated on DNB agar. As shown in Fig. 1a, colonies of bacteria detached from sand grain surfaces which were washed twice appeared intermittently as was observed for the bacteria obtained from a grassland and a paddy soil. The intermittent appearance of colonies became less clear for the bacteria detached after 10 washings and sonication as shown in Fig. 1b and 1c. However, it is noteworthy that as the detachment procedure became stronger, i.e., from washing 2 times to sonication, the organisms which needed a longer

![Graphs showing the increase in the number of bacterial colonies as a function of incubation time.](image)

Fig. 1. Increase in the number of bacterial colonies as a function of incubation time.
(a) The bacteria detached by washing 2 times, (b) washing 10 times and (c) detached by sonication. The dilution rate was 10^4 through (a) to (c).
(d) The number of colonies relative to that formed in the early 3 days. The colony number was divided by the number which appeared in the early 3 days; (○) detached by washing 2 times and (▲) washing 10 times, and (□) by sonication.
time to form visible colonies increased their ratios (Fig. 1d). This seems to reflect that slow-growing organisms attach more firmly to the sand surface.

The total number of colonies formed on DNB agar was $1.4 \times 10^7/(g\ dry\-sand)$ for the sample washed twice and $2.8 \times 10^5/(g\ dry\-sand)$ for that washed ten times. This number increased sharply to $2.0 \times 10^7/(g\ dry\-sand)$ for the bacteria detached by sonication.

DNB agar has been used to isolate bacteria from various natural environments, e.g., grassland\textsuperscript{4,5}, paddy soil\textsuperscript{12} and woodland\textsuperscript{16}, because more cells can form colonies on the medium than on NB agar. The bacteria detached from the river sand grains showed a similar tendency; i.e., the numbers of colonies formed on DNB were about 1.5 to 2 times greater than those formed on NB regardless of the difference in the number or intensity of washing procedures (data not shown).

**Cell surface charge**

We divided the isolates into groups 1, 2 and 3 according to the differences in the colony forming time (tr) being 0–3, 4–7 and 8–13 days, respectively, based on the colony forming curve shown in Fig. 1. It has been reported that the slower growing strains needed a longer time to make the mass of dividing cells large enough to be a visible colony\textsuperscript{4,5}. Thus, we assumed that the growth rates of the isolates from the sand grains decreased in the order from group 1 to 3. Hereafter, we designated the isolates of group 1 as fast-growing strains and those of group 3 as slow-growing strains. As shown in Fig. 2, the isolates belonging to group 3 (slow-growing strains) were less negatively charged at pH 7 except those obtained after 10 washings: There was a significant difference (two-sample t-test, $P<0.05$) between the EPM values of group 1 and 3 for the strains detached by sonication. The fact that the slow-growing isolates are less negatively charged at their cell surfaces has also been reported for the isolates from a grassland\textsuperscript{8} and a paddy soil\textsuperscript{12}.

As pH decreases, microbes tend to have lower levels of negative EPM because the dissociation of functional groups, e.g., carboxylic groups, causing the negative electric charge is depressed\textsuperscript{7}. In more acidic conditions, the microbes may have a positive EPM. Thus, from the change of EPM, as a function of pH, we can estimate a pH value where the EPM value is zero; the pH value is called an isoelectric point (IEP). The relationship between the IEP and the EPM value at pH 7 for the isolates from the sand grains is shown in Fig. 3. Figure 3 shows that the isolates less negatively charged at pH 7 have greater IEP values except for some isolates whose IEP could not be determined because of their negative EPM even in the most acidic condition (pH 2): These exceptional isolates were temporarily plotted as IEP one in Fig. 3.

**Hydrophobicity of cell surface**

Kasahara et al.\textsuperscript{6} found a relation between growth rate and cell hydrophobicity for the isolates from a grassland. They showed that the microbes that needed a longer time to form visible colonies on DNB agar were more hydrophobic at their cell surfaces. This tendency was also observed for the isolates from the surface of sand grains as revealed by hydrophobic interaction chromatography. We divided the isolates into three groups according to their colony appearance time, regardless of the difference in the washing pro-
procedure. Then, the relationship between the group number and the hydrophobicity of the cells was examined. As shown in Fig. 4, as the group number increases, i.e., as the growth rate of the strains decreases, the bacterial cells were retained much more on hydrophobic octyl-Sepharose: There was a significant difference (two-sample t-test, P<0.05) between group 1 and 3.

Cell attachment

As described above, the isolates which needed a longer time to form visible colonies were less negatively charged and more hydrophobic at their surface. Accordingly we examined the relationship between the time to form visible colonies and the cell attachment to a substrate surface. Although the relationship was not particularly clear for cell attachment to a glass surface (Fig. 5a), a tendency was observed for the attachment to a plastic surface. The slower growing strains belonging to group 3 attached to the plastic surface in greater numbers compared with groups 1 and 2, except the isolates obtained after 10 washings (Fig. 5b). We chose isolates from a smaller number of colonies for the 10 times washed fraction compared with the twice washed and sonicated fractions. This may be a reason for the discrepancy in the surface characteristics and attachment behavior of the isolates obtained after 10 washings.

By subtracting the cell number attached to the glass surface from that to the plastic surface, a tendency emerged as shown in Fig. 5c. More isolates attached to the plastic surface than to the glass surface, which was most clearly observed for the bacteria detached after sonication. In these bacteria, slower growers attached to the plastic surface in greater numbers than the fast growing strains.

Discussion

In this study concerning bacterial isolates from river sand grains, we revealed that, compared with fast growing strains, slow-growing strains are less negatively charged (see Fig. 2), more hydrophobic (see Fig. 4) at their surfaces and attach to a plastic surface in greater numbers than to a glass surface (see Fig. 5). This difference in the attachment behavior can be explained in terms of adhesion energy as follows. The surface tension of the glass and the plastic plate was calculated from the contact angle of a droplet (5 µl) of
a hydrophobic and a hydrophilic liquid on the plate by following the method of Busscher et al. 2) and Kasahara et al. 6). The contact angles of $\alpha$-bromonaphthalene were 42.2° ± 0.3° on the glass and the plastic surface, respectively, determined in two independent experiments. The angles of a 0.1 M NaCl solution were 81.1° ± 0.8° and 83.3° ± 0° on the glass and plastic, respectively, in two experiments. From these values we can calculate the surface tension by using a geometric mean approach and Young's equation 1,2,6,15). For the glass surface, the polar component of the surface tension is 6.8 (mJ/m²) and the non-polar component is 25.4 (mJ/m²). For the plastic surface, these are 4.5 and 29.3 (mJ/m²) as the polar and non-polar component, respectively. By using the Fowkes approach 3), we can estimate the adhesion energy, $\Delta G_{adh}$ (mJ/m²), of a microbe to the glass surface as below,

$$\Delta G_{adh} = -61.2 - 0.74(\gamma_{bd})^{1/2} + 9.08(\gamma_{bp})^{1/2}$$

where $\gamma_{bd}$ is the non-polar component and $\gamma_{bp}$ is the polar component of the surface tension of a microbe. The adhesion energy to the plastic surface, $\Delta G'_{adh}$ (mJ/m²), is given as below,

$$\Delta G'_{adh} = -64.7 - 1.49(\gamma_{bd})^{1/2} + 10.06(\gamma_{bp})^{1/2}$$

By subtracting eq. 1 from eq. 2, we can ascertain the difference in the adhesion energy $d\Delta G_{adh}$ (mJ/m²),

$$d\Delta G_{adh} = -3.5 - 0.75(\gamma_{bd})^{1/2} + 0.98(\gamma_{bp})^{1/2}$$

In equation 3, the coefficient of $(\gamma_{bp})^{1/2}$, i.e., +0.98, is positive. This means that the $d\Delta G_{adh}$ value becomes smaller for a more hydrophobic microbe having a smaller value for $\gamma_{bp}$. Therefore, such a hydrophobic microbe can attach much more easily to a plastic surface compared with a hydrophilic microbe. This seems to be the reason for the observation in Fig. 5c where the bacteria isolated by sonication, the slow growing strains, attached to the plastic surface in greater numbers than to the glass surface, compared with the fast growing strains.

In this study we characterized the cell surface of the isolates by another term, the isoelectric point (IEP). The strains showing a greater IEP were less negatively charged at pH 7 as revealed in Fig. 3, and the less negatively charged strains were more hydrophobic as mentioned above. These findings, therefore, reasonably lead to a postulation that the strains having a greater IEP will be more hydrophobic and many more will attach to a hydrophobic surface, e.g., octyl-Sepharose. This was the case for the isolates from the sand grains because the isolates showed a high affinity for the hydrophobic surface as the IEP value increased as shown in Fig. 6.
Recently, it has been reported that hydrophilic polymers on a microbial cell surface may depress the cell attachment due to steric repulsion\textsuperscript{9,10,11,17}. Rijnaarts et al.\textsuperscript{11} have pointed out that an IEP value smaller than 2.8 indicates the presence of a hydrophilic polymer on the cell surface that inhibits the cell attachment to either a hydrophilic or hydrophobic substrate surface. For the isolates from the surface of sand grains, there was no distinct IEP value as Rijnaarts et al. had pointed out (data not shown). However, when the isolates were divided into three groups (IEP less than 2, between 2.1 and 3.0, and greater than 3.1), it was obvious that the strains having a smaller IEP attach less to either the glass or plastic surface than those having a greater IEP, as shown in Fig. 7. It is also noteworthy that the number of cells attached to the plastic surface was greater than that to the glass surface, especially for the strains having an IEP greater than 3.1, whose cells have a hydrophobic surface as shown in Fig. 6. This may reflect the fact that the strains having a smaller IEP cannot attach firmly to a substrate surface because of the steric repulsion caused by the hydrophilic polymer on their surfaces.

In this study, we obtained data showing that slow-growing isolates were more hydrophobic and less negatively charged at their surfaces and could attach in greater numbers to a hydrophobic than to a hydrophilic surface. The slow-growing strains showed a higher population frequency among the isolates having IEP values greater than 3.1 compared with the isolates having smaller IEP values as shown in Fig. 8. Moreover, these slow-growing strains increased their ratio in the bacteria attaching more firmly to the sand surface (Fig. 1d). These findings may indicate that slow-growing bacteria have the surface characteristics, as revealed in this study, which enable the cells to attach more firmly to a substrate surface for survival in competition with fast-growing bacteria.

![Figure 6](image6.png)

Fig. 6. Relationship between isoelectric point (IEP) and the percentage retention of the bacterial cells on octyl-Sepharose.

Bars are standard deviations.

![Figure 7](image7.png)

Fig. 7. The frequency distribution in the number of attached cells to (a) the glass and (b) the plastic surface.

The isolates from the sand grain surfaces were divided into (■) the strains whose IEP was less than 2, (□) between 2.1 and 3.0, and (■) greater than 3.1. The ratio of the strains belonging to a certain range of the attached cell number, to the total number of strains in each IEP group is shown.
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