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A toluene-degrading bacterium, *Acinetobacter* sp. Tol 5, shows noteworthy adhesiveness mediated by two types of cell appendages. In this study, we obtained a less-adhesive mutant, T1, which lost both types of appendages, and investigated how the cell appendages affect the adhesion properties of this useful bacterium for environmental technology. Wild-type cells attained irreversible adhesion to polyurethane carriers within 30 s, while adhesion of T1 cells was still reversible at that time. While T1 showed decreased adhesion with decreasing ionic strength and did not adhere at all at 0.015 mM, adhesion of the wild type was fully independent of ionic strength. *Acinetobacter* sp. Tol 5 was also found to be not motile. Our results suggest that through the long distant interaction mediated by the appendages between the cells and surfaces, Tol 5 cells can attain irreversible adhesion very quickly without approaching the vicinity of the substratum.

Key words: microbial adhesion; *Acinetobacter*; cell appendage; Derjaguin-Landau-Verwey-Overbeek (DLVO) theory

Microbial adhesion is the initial step in colonization and biofilm formation. It is detrimental to both human life and industrial processes, causing infection and contamination by pathogens, dental decay, and biofilm formation on industrial facilities and ship’s hulls, but it can also be beneficial in some environmental bioprocesses and in agriculture. Recently, microbial adhesion that is the strongest ever measured for biological adhesives was reported, and attracted our attention with expectations for applications of a powerful bond. To date, many studies have been reported on the mechanism of microbial adhesion of pathogenic strains such as *Pseudomonas aeruginosa*, *Staphylococci*, *Enterococci*, *Serratia* species, and *Escherichia coli*. Even less than pathogenic strains, the adhesion of environmental species that cause problems, such as marine organisms and *Pseudomonas* species, has also been studied, but there is little information about useful strains forming applicable biofilm for the decontamination of toxic chemicals.

Recently, *Acinetobacter* sp. Tol 5, a highly adhesive bacterium capable of degrading toluene, was isolated. The adhesiveness of this toluene degrader is noteworthy. The inner walls of plastic tips and pipettes are coated with the cells at once by just sampling of them. We found two morphological types of appendages that have the function of connecting the Tol 5 cells to a substratum, an anchor-like appendage and a peritrichate fibril-type appendage. Contemporary electron microscopy techniques revealed that the former extends straight to the substratum without branching and tethers the cell body at its end from distances of several hundred nanometers, whereas the latter attaches to the substratum in multiple places, fixing the cell at shorter distances. Cell appendages have been shown to be involved in bacterial adhesion, but the nature of the long-distance interactions between bacterial cells and a substratum mediated by appendages, such as the anchor in *Acinetobacter* sp. Tol 5, has not yet been reported. Hence we studied the way the cell appendages on the Tol 5 cells affect adhesion properties.

Materials and Methods

**Strains and cultivation.** *Acinetobacter* sp. Tol 5 was grown in 20 ml of basal salt (BS) medium3 supplemented with 10 µl of toluene (the equilibrated aqueous toluene concentration was 164 µg/ml) in a 100-ml Erlenmeyer flask. To the flask, four pieces (1.15 × 1.15 × 1.00 cm) of sponge carrier made of polyurethane, with specific surface area, density, and porosity of 12
cm\(^2\) / cm\(^3\), 0.115 g/cm\(^3\) and 73.8% respectively, were added. The flask was capped with a rubber stopper and shaken at 115 rpm at 28 °C. Escherichia coli S17-1 carrying the transfer genes of RP4 on its chromosome and the plasmid pSUP102-Gm::Tn5-B30 was kindly provided by Dr. Kensuke Furukawa of Kyushu University. This plasmid harbors a gentamicin-resistance gene, an RP4-specific mob site, and a transposon Tn5 derivative, Tn5-B30, containing a tetracycline-resistance gene.\(^5\) E. coli S17-1 was grown in Luria-Bertani (LB) medium supplemented with tetracycline (12 μg/ml) and gentamicin (15 μg/ml) at 37 °C. Pseudomonas aeruginosa PAO1 was grown in 20 ml of M8 medium\(^7\) supplemented with 0.2% (w/v) glucose and 0.05% (w/v) glutamate at 28 °C. Escherichia coli O157: H7 (ATCC43888) was grown in 20 ml of LB medium at 37 °C.

**Adhesion and detachment tests.** Cells were cultivated until stationary phase in the presence of polyurethane. The cells were harvested and resuspended in BS medium to an optical density of 0.5 at 660 nm (OD\(_{660}\)). Four pieces (6,400 mm\(^2\) total surface area) of the fresh polyurethane carriers were added to 20 ml of the cell suspension in a 100-ml Erlenmeyer flask. The cells and polyurethane carriers were incubated at 28 °C with shaking at 115 rpm in the absence of a carbon source so that the bacterium would not grow during incubation. During incubation, the change in the OD\(_{660}\) value of the aqueous phase was analyzed. The cell concentration corresponding to an OD\(_{660}\) of 1.0 was 8.4 × 10\(^8\) cells/ml. The number of cells adhering to the carrier was calculated from the initial cell concentration and the total surface area of the polyurethane carriers. Because the initial cell concentration, the total surface area of the carrier, and the volume of the cell suspension were 4.2 × 10\(^8\) cells/ml (OD\(_{660} = 0.5\)), 6.4 × 10\(^3\) mm\(^2\), and 20 ml respectively, the specific number of adhering cells obtained from 100% of the adhesion ratio was 1.3 × 10\(^7\) cells/mm\(^2\).

For a detachment test, the polyurethane carriers, to which the bacterial cells partially adhered, were collected at 30 s during the adhesion test. The carrier was transferred to 20 ml of fresh pure water in a flask and subjected to further incubation with shaking at 115 rpm. During incubation, the change in the OD\(_{660}\) value of the aqueous phase was analyzed to estimate the number of cells detached from the polyurethane. After conversion of the OD\(_{660}\) value into cell numbers, the specific number of adhering cells remaining on the polyurethane was calculated as described above.

**Transposon mutagenesis and selection of less-adhesive mutants.** To generate transposon insertion mutants of Acinetobacter sp. Tol 5, wild-type strain Tol 5 (WT) was conjugated with a donor strain, E. coli S17-1, harboring the suicide plasmid vector pSUP102Gm::Tn5-B30 on a filter at 28 °C for 20 h. After conjugation, the plasmid was transferred to the recipient cells (Tol 5), and subsequently Tn5-B30 was inserted into the chromosome of the recipient cells. Transformants can be readily selected on a plate containing tetracycline for the marker gene on Tn5-B30, but it is difficult to select less-adhesive mutants from random transformants on a plate. Hence we established a new method for efficient selection of less-adhesive mutants. After conjugation, the filter with grown colonies was washed in 1 ml of 0.85% (w/v) NaCl solution to recover all cells from it. A portion (400 μl) of the cell suspension was used to inoculate 20 ml of BS medium supplemented with toluene (164 μg/ml) as the sole carbon source and tetracycline (12 μg/ml). The cells were grown at 28 °C in the presence of the polyurethane carriers. When WT cells were grown under the same conditions, except for the absence of tetracycline, the concentration of planktonic WT cells in the bulk medium was 2.0 × 10\(^4\) CFU/ml (the background value). Therefore, the culture broth of the mutants was diluted more than 10\(^{-4}\) with BS medium, and subsequently 100 μl of the diluted culture broth was spread onto a BS plate containing 30 μg/ml of tetracycline. The plate was incubated at 28 °C under a supply of toluene vapor. The colonies grown on the plate were examined for insertion of the tetracycline-resistance gene (tet A) by PCR to confirm that they had been generated by the integration of Tn5-B30 into their chromosome.

**Microbial-adhesion-to-hydrocarbons (MATH) test.** Cells were harvested by centrifugation (4,000 rpm, 10 min), washed with sterile water, and resuspended in BS. The optical density (OD\(_{660}\)) of the cell suspension was measured. To 1.0 ml of the cell suspension, 200 μl of hexadecane was added. After vigorous shaking by vortex for 30 s, OD\(_{660}\) of the aqueous layer was measured. The reduced percentage of OD\(_{660}\) was referred to cell surface hydrophobicity (eq. 1).

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\text{MATH} \, (\%) = \frac{\text{OD}_{660}-\text{before} - \text{OD}_{660}-\text{after \, treatment}}{\text{OD}_{660}-\text{before}} \times 100
\]

**Electron microscopy.** All samples were obtained from cultures grown to stationary phase in the presence of the polyurethane carriers, with toluene as the sole carbon source. All procedures for pretreatment of cells and for field emission scanning electron microscopy (FE-SEM) were carried out as described in a previous paper.\(^4\) Transmission electron microscopy (TEM) of cells that were negatively stained with phosphotungstic acid was also performed, as described previously.\(^3\)

**Swarming and swimming tests.** Swarming agar plates were prepared using M8 salt supplemented with 0.2% (w/v) glucose and 0.05% (w/v) glutamate, and solidified with 0.6% (w/v) agar.\(^7\) Cells from single colonies were inoculated onto the middle of the swarming plates.
with a toothpick. *P. aeruginosa* PAO1 and *E. coli* O157: H7 (ATCC43888) were incubated at 37 °C for 2 d. *Acinetobacter* sp. Tol 5 was incubated at 28 °C for 2 d in a desiccator containing gas-phase toluene at a concentration of 0.2% by volume. Swimming plates were made of LB medium containing 0.25% (w/v) agar and inoculated as described above. *P. aeruginosa* PAO1 and *E. coli* O157: H7 were incubated at 37 °C for 16 h. *Acinetobacter* sp. Tol 5 was incubated at 28 °C for 16 h.

**Results and Discussion**

**Obtaining a less-adhesive mutant that lost the cell appendages**

To investigate the effect of the presence of the cell appendages on the adhesion properties of strain Tol 5, less-adhesive mutants that lost them were obtained by transposon mutagenesis coupled with an efficient method, which was established in the present study. Among the several resulting mutant strains, T1 showed similar growth features to WT, but T1 cells were individually dispersed in the culture medium even in the presence of polyurethane carriers (data not shown). According to the adhesion test, only 16% of the T1 cells adhered to the polyurethane (adhesiveness: \(2 \times 10^5\) cells/mm\(^2\)), while almost all of WT cells adhered (adhesiveness: \(1.3 \times 10^6\) cells/mm\(^2\)) (Fig. 1). According to the MATH test, however, the cell surface hydrophobicity of T1 was 87.1%, while that of WT was 91.8%. This indicates that the less-adhesive mutant showed similar hydrophobicity to WT cells.

T1 cells adhering to the polyurethane surface during growth were observed by FE-SEM. This observation revealed that T1 cells lacked both types of appendages (Fig. 2B). The lack of these appendages was also confirmed by TEM coupled with negative staining with phosphotungstic acid (Fig. 2C). Thus we obtained a less-adhesive mutant that had lost the cell appendages.

**Reversibility of cell adhesion**

Bacterial adhesion is thought to be a two-phase process comprising an initial, loosely attached, reversible state (phase one), and a time-dependent, more strongly attached, irreversible state (phase two) (the two-step adhesion model).\(^1\,^5\,^8\,^9\) We examined the effect of the cell appendages on the reversibility of the adhesion of Tol 5 cells using WT and T1.

First, \(8.3 \times 10^9\) of Tol 5 cells grown on toluene were incubated at room temperature for 60 min in 20 ml of fresh BS medium in the presence of pieces of polyurethane carrier with a total surface area of 6,400 mm\(^2\) (the adhesion test). After 30 s of incubation in the adhesion test, the polyurethane carriers to which the bacterial cells were partially adhered were immediately collected, transferred to fresh pure water, and subjected to further incubation for up to 60 min. During this detachment test, the WT cells never detached (Fig. 3), which implies that they had already attained irreversible adhesion within 30 s of incubation with the polyurethane carriers. If the adhesion had been reversible, a proportion of the adhering cells would have detached from the polyurethane surface into the fresh pure water by cell motion with thermal kinetic energy. In fact, a proportion of the adhered cells of T1 detached from the polyurethane carriers. Finally, the fraction of detached T1 cells almost reached a plateau at a level of 45% of the cells that had initially adhered to the surface. This level is thought to represent the adsorption-desorption equilibrium and/or the transition from the reversible to the irreversible state, because this transition is expected to continue during incubation in the detachment test. These results suggest that the cell appendages afford Tol 5 cells the capability of instant and irreversible adhesion.

The two-step adhesion model is based on Derjaguin-Landau-Verwey-Overbeek (DLVO) theory,\(^8\) which originally described the interaction of a colloidal particle with a surface as a summation of van der Waals and Coulomb interactions.\(^11\) Because the surfaces of cells and abiotic materials generally have a net negative charge in an electrolyte solution, repulsive electrostatic energy is caused by overlap of the electrical double layers of a bacterial cell and a substratum.\(^1\,^8\,^12\,^13\) This repulsive energy increases as the ionic strength of a suspension medium decreases because shielding of the surface charges by the ions on the electrical double layers lessens. At low ionic strengths, an energy barrier, which bacterial cells cannot surmount by swimming or Brownian motion, is formed with an energy minimum on either side.\(^8\,^13\,^16\,^17\) The primary energy minimum at the side closer to a substratum is deep enough to capture a bacterial cell irreversibly, but bacterial cells cannot reach that side because of the energy barrier. Therefore,
bacterial cells adhere reversibly at the secondary shallow energy minimum at the side farther from the substratum (first step). Then the cells attain irreversible adhesion by bridging with appendages, such as pili and flagella, or exopolymeric substances (EPS), which can pierce the energy barrier due to their small radii (second step). However, WT cells attained irreversible adhesion instantaneously even in pure water. Hence it was supposed that the cell appendages caused a deviation in the adhesion of Tol 5 cells from the conventional DLVO theory.

Dependence of cell adhesion on ionic strength
Many researchers have found a link between decreasing bacterial adhesion and decreasing ionic strength that is consistent with DLVO theory. Hence the effect of ionic strength ($I$) on the adhesion of the Tol 5 cells was investigated. The cells were harvested, washed with pure water, resuspended in a dilution series of BS medium ($I = 340 \text{mM}$) and pure water, and then subjected to 30-min adhesion tests. As shown in Fig. 4, the number of adhering T1 cells was $2.5 \times 10^5 \text{cells/mm}^2$ at an ionic strength of 340 mM. This number decreased with decreasing ionic strength until, ultimately, T1 cells did not adhere at all at an ionic strength of 0.015 mM. In contrast, WT cells retained high adhesion to the polyurethane despite the decreasing ionic strength. These observations imply that WT cell adhesion via the long appendages does not follow the trends predicted by DLVO theory, whereas T1 cells, which lack these long appendages, adhere in a manner consistent with DLVO theory.

Role of the appendages in cell adhesion
Cell motility caused by cell appendages such as flagella facilitates adhesion. To investigate the possibility that cell motility given by the appendages is one of the reasons for the different adhesiveness of WT and T1 cells, cell motility was examined on specific agar plates. Control tests were carried out using P. aeruginosa PAO1, which has swimming motility due to flagella and swarming motility due to type IV pili, and E. coli O157: H7, which has only swimming motility due to flagella. These motility tests clearly revealed that Acinetobacter sp. Tol 5 possesses neither swimming nor swarming motility, whereas the expected motilities of P. aeruginosa PAO1 and E. coli O157: H7 were confirmed (Fig. 5). T1 cells also showed no motility (data not shown). Therefore, it was confirmed that the appendages on Tol 5 cells work for adhesion but not through motility.

The images of the adhesion of T1 cells to the polyurethane surface (Fig. 2B) clearly showed that the cells tended to accumulate at stagnant sites such as grooves, cracks, and voids on the surface via a hydrodynamic effect, because Tol 5 does not have cell motility. The cells that accumulate at these types of sites are sheltered from shear forces, resulting in an increased...
chance of remaining at the secondary energy minimum until the irreversible phase of the adhesion process is attained. In contrast, WT adheres to the whole surface, covering it in the form of multi-layer cell clusters. The long-distance connection mediated by the appendages appears to dispense with the need for the cell body to be conveyed to the vicinity of the substratum for adhesion. Thus, adhesion of an environmentally applicable bacterium, Acinetobacter sp. Tol 5, changes the general concept of bacterial adhesion, which was previously thought to be initiated only by cells located in close proximity to the substratum. However, because both types of appendages were lost on T1 cells, whether one or both of them is responsible for the particular and interesting adhesion process of the Tol 5 cells remains to be studied, as well as the reason for the simultaneous deficiency. We are now analyzing a DNA region of T1 at the Tn5-insertion site.

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