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Evaluation of adhesiveness of *Acinetobacter* sp. Tol 5 to abiotic surfaces

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The toluene-degrading bacterium *Acinetobacter* sp. Tol 5 shows a highly adhesive and autoagglutinating nature through cell surface nanofibers. In the present study, various aspects of the adhesiveness of Tol 5 cells were evaluated, namely, the association with biofilm formation, the specificity, and the effects of additives. During growth, Tol 5 and *Pseudomonas aeruginosa* PAO1 cells showed high capacities to form biofilms. However, resting PAO1 cells barely adhered to a polystyrene (PS) surface, while a large number of resting Tol 5 cells rapidly adhered. This implies that Tol 5 cells are intrinsically adhesive and that their initial attachment ability is quite high and distinguishable from their ability to form biofilm. This high adhesiveness of Tol 5 cells was considered to be nonspecific because the cells adhered to various material surfaces from hydrophobic plastic surfaces to hydrophilic glass and metal surfaces. However, Tol 5 cells were found not to be adhesive to *Escherichia coli* cells. Although Tol 5 cells were capable of interspecies interaction and coagglutination with *E. coli* cells, at the surface of cell clumps, *E. coli* cells localized, suggesting that they disturbed autoagglutination of Tol 5 cells. Tol 5 has a hydrophobic cell surface. However, the addition of nonionic surfactant Triton X-100 and bovine serum albumin increased the adhesion of Tol 5 cells to a PS surface, in contrast to previous reports of hydrophobic bacteria. The results highlighted the interesting features of adhesiveness of Tol 5 cells.

[**Key words:** Bacterial adhesion; Biofilm; *Acinetobacter*; Bacterionanofiber; Agglutination]

Biofilms are accumulated biomasses of microorganisms and extracellular materials on solid surfaces and have attracted much attention in various fields. On the one hand, biofilms can be detrimental to both human life and industrial processes, for example, causing infection, pathogen contamination, and slime formation; on the other hand, they can be beneficial in environmental technologies and bioprocesses (1). Generally, bacteria tend to form biofilms on biotic and/or abiotic surfaces (2), which is a complicated process consisting of two phases: an initial adhesion phase and the subsequent biofilm development phase. Each phase further consists of multiple steps. In the adhesion phase, bacterial cells first approach the substratum by intrinsic motility, Brownian motion, convection, gravitation, and so on. Then the cells reversibly interact with the substratum through physicochemical interactions such as van der Waals, electrostatic, and hydrophobic interactions. Subsequently, the cells attain irreversible adhesion by frequently using lipopolysaccharide and bacterionanofibers, which are filamentous cell appendages of several nanometers in diameter, such as pili and flagella, for bridging between the cells and the substratum. In the biofilm development phase, while growing and secreting exopolymeric substances (EPS), the cells form microcolonies and then gradually develop biofilms.

Acinetobacter sp. Tol 5, which is a toluene-degrading bacterium isolated from a biofiltration system, exhibits an autoagglutinating nature and high adhesiveness mediated by bacterionanofibers (3–6). Even simple cell sampling results in the instant coating of inner walls of plastic tips and glass pipettes with cells. We initially identified two morphologically different nanofibers on the Tol 5 cell surface: peritrichate pilus-like and non-peritrichate anchor-like nanofibers (4). However, improved electron microscopy techniques later enabled discrimination of at least three kinds of peritrichate nanofibers on Tol 5 cells, two of which were deduced to be type 1 and Fil pili (7).

In the present study, various aspects of the adhesiveness of Tol 5 cells were evaluated, namely, the association with biofilm formation, the specificity, and the effects of additives. The results highlighted the interesting features of Tol 5 cell adhesion which are not shared by *Pseudomonas aeruginosa* PAO1, a representative bacterial strain with biofilm formation ability, and hinted at the mechanism of interaction between Tol 5 cells and surfaces through the nanofibers.

MATERIALS AND METHODS

Bacterial strains and cultivation *Acinetobacter* sp. strains Tol 5 and ADP1 (8) were grown in Luria-Bertani (LB) medium at 28°C. *P. aeruginosa* PAO1 cells (9), kindly provided by Dr. Nobuhiko Nomura of University of Tsukuba, were grown in LB medium at 37°C. *Escherichia coli* DH5α cells and transformants harboring the pAcGFP1 plasmid (Clontech) were grown at 37°C in LB medium, supplemented with ampicillin (100 μg/mL) for the transformant. Cell surface hydrophobicity of bacterial strains was analyzed by the microbial-adherence-to-hydrocarbon (MATH) tests, as described previously (3).

Biofilm formation assay The biofilm formation assay was performed according to the method of O'Toole and Kolter (10). Briefly, *Acinetobacter* sp. Tol 5 and *P. aeruginosa* PAO1 were grown overnight. The overnight cultures were inoculated into LB medium in 96-well polyvinylchloride (PVC) plates (2595; Coster) at an OD_{660} of ~0.01 and incubated

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at 28°C for Tol 5 and 37°C for PAO1. After incubation for a certain number of hours, the wells of the plates were washed with tap water twice and biofilms that formed on the inner walls of the wells were stained with 125 μL of 0.1% crystal violet for 15 min. After the crystal violet solution was gently removed, the wells of the plates were washed with tap water twice, air-dried, and then photographed. For quantification of the biofilms stained with the dye, 200 μL of 99.5% ethanol was added to each well and transferred to a new tube. After this procedure was repeated once and the ethanol solution was collected into the same tube, the volume was adjusted to 1 mL with fresh 99.5% ethanol and the absorbance was measured at 590 nm (A_{590}) on a spectrophotometer (U-2810; Hitachi).

Adherence assay Bacterial strains were pre-cultivated in 2 mL of LB medium overnight at 28°C for *Acinetobacter* strains or at 37°C for *P. aeruginosa* and *E. coli*. The overnight culture was diluted to 1:100 with fresh LB medium and grown for 6 h. The bacterial cells were harvested by centrifugation, rinsed with BS-N medium (11), and resuspended in the same medium at an OD_{660} of ~0.5. Cell suspension (1 mL for a 48-well plate, 100 μL for a 96-well plate, and 2.0 mL for a sample cup) was placed into each well of 48-well polystyrene (PS) plates (3548; Costar), 96-well plates made from PS (353072; Becton, Dickinson and Company), polypropylene (PP) (3364; Costar), PVC (2595; Costar), or glass (FB-96; Nippon Sheet Glass Co., Ltd.), or into a sample cup made of stainless steel (SUS) (92-0456-4; SANSYO). Prior to the assays in the glass plates, Piranha solution (H_2O_2/H_2SO_4 = 1:3) was placed into each well and incubated for 1 h at room temperature to remove organic contaminants on the glass surface. The glass plate was washed with pure water and instantly dried at 60°C. The cell suspension was removed after incubation for a certain number of hours at 28°C (Tol 5 and ADP1) or 37°C (PAO1 and DH5α), and the wells or cups were washed twice with BS-N medium. Cells adhering to the inner walls of the wells or cups were stained with 1% crystal violet solution for 15 min, and washed three times with BS-N medium. Finally, the stain was dissolved in 99.5% ethanol (1 mL for the 48-well plates, 200 μL for the 96-well plates, and 2.5 mL for the SUS cups), and the A_{590} was measured for the quantification of adherence. Statistical analysis was performed by Student's *t*-test.

When the effects of the presence of Triton X-100 or bovine serum albumin (BSA) on bacterial cell adhesion and autoagglutination were investigated, they were added to BS-N medium in various concentrations to wash the cells and wells and to prepare the cell suspensions.

Agglutination assay Agglutination was quantified by a tube-settling assay (12–14). Bacterial cell suspensions were prepared by the same procedure as the adherence assay mentioned in the previous section. In the coagglutination assay, equal volumes of Tol 5 and *E. coli* cell suspensions were mixed. Cell suspension (8 mL) at an OD_{660} of ~0.5 was placed into a test tube, which was then left to stand at room temperature to allow sedimentation of cell clumps. The suspension was sampled from the top of the test tube, and the OD_{660} was measured. The autoagglutination ratio was calculated from the change in OD_{660} using the following equation:

$$\text{Autoagglutination ratio } (\%) = 100 \times (\text{Initial } OD_{660} - OD_{660} \text{after standing})/\text{Initial } OD_{660}$$

For microscopic analysis of the coagglutination of Tol 5 and *E. coli* cells, *E. coli* DH5α was transformed with pAcGFP1 for labeling with green fluorescent protein (GFP). Bacterial cells were sampled from the supernatant or the sediment after 4 h of incubation in the tube-settling assay of the mixed cell suspension of Tol 5 and *E. coli* cells. The sample was placed onto a gelatin-coated plate, fixed with 4% paraformaldehyde solution for 15 min at room temperature, washed with pure water three times, and observed by confocal laser scanning microscopy (FV1000D; Olympus).

RESULTS AND DISCUSSION

Distinction between biofilm formation and attachment ability Although the high adhesiveness of Tol 5 to solid surfaces has been shown, the association between adhesiveness and biofilm formation ability has never been examined. Therefore, biofilm formation ability was compared between Tol 5 and *P. aeruginosa* PAO1, which is a representative strain that forms biofilms. Biofilms of both strains were barely observed during the beginning of the assay (0.5–2.0 h), but became visible during the growth period (4–10 h) (Fig. 1). Although the masses of formed biofilms were similar, there were differences in the biofilm formation process between the two strains. PAO1 biofilm developed sharply at around 4 h of incubation, reached the maximum at 6 h, and thereafter oscillated probably due to the release and diffusion of cells from the biofilm. Tol 5 continuously developed biofilm throughout the 10 h assay. While PAO1 biofilm formed at the interface between the liquid medium and air, Tol 5 biofilm formed over the whole well surface in contact with the medium, particularly at the bottom and the interface. This difference might be due to variations in adhesiveness, the autoagglutinating property of forming large cell clumps, and intrinsic motility; PAO1 has swimming, twitching, and swarming motility, but Tol 5 is nonmotile (5).

Although we previously demonstrated that *Acinetobacter* sp. Tol 5 adhered to a PS plate for 2 h incubation in non-growth conditions (7),

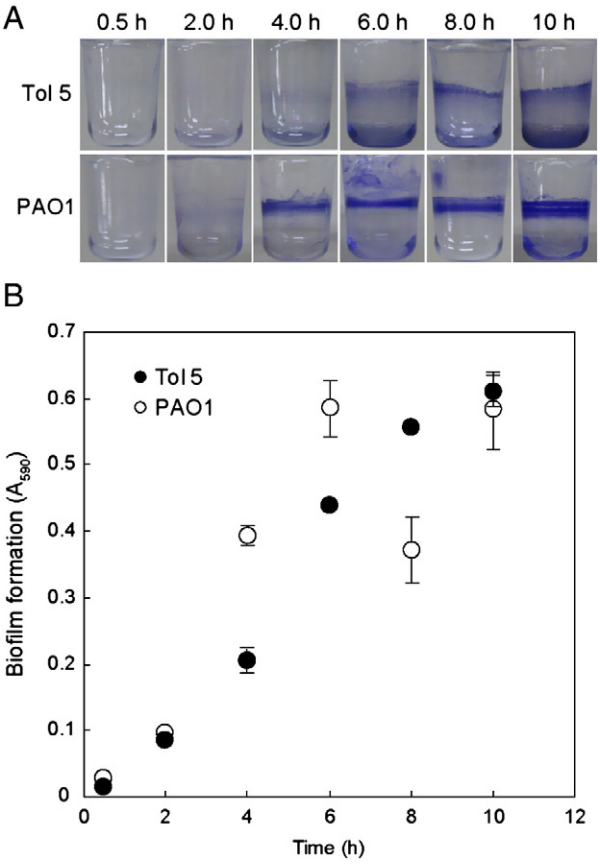

FIG. 1. Time courses of biofilm formation on PVC plates by *Acinetobacter* sp. Tol 5 and *P. aeruginosa* PAO1. (A) Each strain was grown in LB medium in 96-well PVC plates and biofilms that formed were stained with crystal violet. (B) The biofilms stained with crystal violet in panel A were quantified by measuring the absorbance at 590 nm (A_{590}) of an ethanol solution used to dissolve the stain. Data are expressed as mean ± SEM (n = 6).

we compared the time course of Tol 5 adhesion with that of PAO1. Bacterial cells grown to the stationary phase in LB medium were suspended in a basal salt medium without carbon and nitrogen sources for the adherence assay. In these resting cells, growth, *de novo* protein synthesis, and EPS production should be restrained during the assay. Resting cells of Tol 5 adhering to the PS plate were visible by staining with crystal violet, even after 0.5 h (Fig. 2A). The mass of adherent Tol 5 cells increased with time and became almost constant after 2 h (Fig. 2B). In contrast, PAO1 resting cells barely adhered to the PS surface, although faint adhesion was detected by colorimetry for an initial short period.

Thus, PAO1 cells showed high capacity to form a biofilm during growth, despite the non-adhesive property of resting cells. This suggests that PAO1 cells express adhesive factors, such as EPS that contains a variety of biopolymers including polysaccharides, proteins, and DNA, during growth. In contrast, the adhesiveness of Tol 5 cells, that is, the capability of a large number of the cells to rapidly adhere to a solid surface, was found to be independent of growth. This implies that Tol 5 cells are intrinsically highly adhesive and that the initial attachment ability is quite high and distinguishable from biofilm formation ability. This unique adhesive nature of Tol 5 cells is supposed to be attributed to nanofibers that the cells have on their surface. A 2 h period of incubation of resting Tol 5 cells provided enough time for the maximum level of adhesion, thus incubation was conducted for 2 h in further adherence assays.

Nonspecificity of Tol 5 cell adhesion In previous work, we reported that Tol 5 cells efficiently adhere to polyurethane foam (5). In addition, we observed that Tol 5 cells also adhere to hydrophilic