A sticky bacterium versus antiadhesive surfaces: The adhesion preference of bacteria expressing trimeric autotransporter adhesin

Short title: A sticky bacterium versus antiadhesive surfaces

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Abstract

While microorganisms have evolved to adhere and form biofilms on surfaces, various materials with antiadhesive surfaces have been developed. The Gram-negative bacterium *Acinetobacter* sp. Tol 5 exhibits high adhesiveness to various surfaces through AtaA, one of trimeric autotransporter adhesins (TAAs). We examined the adhesion of Tol 5 and other bacteria expressing different TAAs to antiadhesive surfaces. The results highlighted Tol 5's stickiness through AtaA, which enables cells to adhere even to antiadhesive materials including polytetrafluoroethylene with a low surface free energy, a hydrophilic polymer brush exerting steric hindrance, and mica with an ultrasmooth surface. Tol 5 cells also adhered to a zwitterionic 2-methacryloyloxyethyl-phosphorylcholine-polymer-coated surface but were exfoliated by a weak shear stress, suggesting that exchangeable bound water molecules contribute to AtaA's interaction with materials.

Introduction

Pathogenic bacteria, like viruses, cause infectious diseases, and the threat is reminded by the COVID-19 pandemic. However, bacteria are not as much of a threat as viruses because antibiotics are effective against them. This is changing, however, with the emergence of antibiotic-resistant bacteria. The global expansion of multidrug-resistant bacteria has become a clinical problem (*1*), and the threat of bacterial infection might come back in the near future. The overuse of antibiotics amplifies the opportunity for resistant bacteria to emerge and spread (*2*). The increased antibiotic use during this COVID-19 pandemic could also increase the threat of resistant bacteria (*3*). As an alternative to antibiotics, antiadhesive (antibiofouling) surfaces have drawn intensive research interest because bacterial adhesion is the initial step of infection by pathogens and biofouling of equipment (*4-7*). As a result of extensive efforts, various antiadhesive surfaces have been developed and characterized, such as fluoropolymers, polymer brushes, highly hydrophilic zwitterionic polymers, and ultrasmooth or nano/micro-topographical patterned surfaces (*8-12*).

Acinetobacter sp. Tol 5, which is a toluene-degrading bacterium that we previously isolated from a biofiltration system, exhibits autoagglutination and high adhesiveness to solid surfaces (*13, 14*). Tol 5 cells quickly adhere to various material surfaces from hydrophobic plastics to hydrophilic glasses and metals independently of biofilm formation (*13*). This characteristic nonspecific adhesiveness of Tol 5 cells is mediated by AtaA, a member of the trimeric autotransporter adhesin (TAA) family (*15-17*). TAAs are outer membrane proteins of Gram-negative bacteria and have been well-studied as virulence factors because each TAA shows an ability to bind to biotic molecules of mammalian host cells and occasionally to some kinds of abiotic surfaces (*18, 19*). Although they have a variety of lengths from several hundreds to several thousands of amino acids, they have a common structure that includes an N-terminal passenger domain (PSD), which is secreted onto the cell surface and is responsible for its function, and a C-terminal transmembrane domain, which anchors the PSD onto the outer membrane (*19*). AtaA is one of the largest TAAs consisting of 3,630 amino acids but shares common structural features with other TAAs, (*15, 20*). However, there have been no reports of TAA-mediated adhesion similar to Tol 5 cells through AtaA in terms of nonspecificity and high stickiness.

In a proverb known as the "shield-spear contradiction" derived from an ancient Chinese text *Han Feizi*, a merchant first boasts that, "this shield is strong enough to prevent anything," and then, "this spear is sharp enough to pierce anything." In response to the merchant's boasting, one person from the crowd asks the merchant, "What would happen if you attack your shield with your spear?" The merchant could not answer. Similarly, we also don't know what would happen if highly adhesive Tol 5 cells encounter an antiadhesive surface. In this study, we investigated the interaction of Tol 5 and some other TAA-expressing bacterial cells with various surfaces including antiadhesive surfaces that have different repelling mechanisms.

Results

Outstanding adhesiveness mediated by AtaA compared with other TAAs

First, we compared the adhesiveness of Tol 5 and its Δ*ataA* mutant (negative control) with that of *Yersinia enterocolitica* and *Bartonella henselae* by shaking each cell suspension in the presence of a polyurethane support for 30 min. These Gram-negative bacteria have also been reported to adhere to abiotic surfaces through their TAAs (*18*), YadA and BadA, respectively. The production of these TAAs was confirmed by western blotting (see Supplementary Figure S1). The result showed the overwhelming stickiness of cells expressing AtaA compared with that of cells expressing the other TAAs (Fig. 1). Most of the Tol 5 cells adhered to the support and the cell suspension became abundantly clear. In contrast, the cell suspensions of *Y. enterocolitica*, *B.*

henselae, and Tol 5 Δ*ataA* mutant remained cloudy, which indicated that many of the cells did not adhere to the polyurethane support.

Next, we quantified the adhesiveness of bacterial cells that express TAAs to various material surfaces. Cell suspensions were placed and incubated on polystyrene (PS), glass, stainless steel, and polytetrafluoroethylene (PTFE, known as Teflon) surfaces for 10 min. Non-adhering cells were removed by washing with a fresh medium and the adhered cells on the material surface were quantified by crystal violet staining. As shown in Figure 2, in a short time (10 min), Tol 5 could adhere to not only PS, glass, and stainless steel but also to PTFE, which has antiadhesive properties derived from its low surface energy (*8*). In contrast, Tol 5 Δ*ataA* mutant and *Y. enterocolitica* hardly adhered to all the material surfaces. Although *B. henselae* showed measurable adhesiveness, the amount of adhered cells was much smaller than that of Tol 5. These results quantitatively demonstrated that Tol 5 cells exhibit remarkably higher adhesiveness to various material surfaces through AtaA than bacterial cells expressing other TAAs.

AtaA versus antiadhesive surfaces

To investigate whether Tol 5 cells adhere to various other antiadhesive surfaces in addition to PTFE, we performed adhesion assays with mica, poly(oligo(ethylene glycol) methyl ether methacrylate) (poly(mOEGMA)) brush, and 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer surfaces. Mica is a phyllosilicate mineral of aluminum and potassium, and its surface after cleaving is atomically flat (*21*). A poly(mOEGMA) brush is a neutral hydrophilic polymer brush and exerts steric repulsion (*10*). An MPC polymer is a zwitterionic hydrophilic polymer and possesses a high free water fraction (*22*). These surfaces have been reported to have antiadhesive properties against bacterial cells (*9, 11, 23*). After incubation of bacterial cells on the antiadhesive surfaces for 10 min, Tol 5 cells adhered to PTFE but not to the mica, poly(mOEGMA) brush, and MPC polymer surface (Fig. 3A). After incubation for 2 h, Tol 5 cells adhered to not only PTFE but also to the mica and poly(mOEGMA) brush surface, but hardly adhered to the MPC polymer (Fig. 3A). In contrast, *B. henselae* adhered to PTFE and mica but not to the poly(mOEGMA) brush and the MPC polymer (Fig. 3B). These results emphasize that Tol 5 cells were the only cells that adhered to the poly(mOEGMA) brush and showed that even sticky Tol 5 cells hardly adhered to the MPC polymer under these experimental conditions.

Cell behavior caused by AtaA on a MPC polymer surface

To investigate how the MPC polymer repels Tol 5 cells, we observed the behavior of Tol 5 cells on the polymer surface by using a flow cell system with a square glass tube (Fig. 4A) (*24*). The glass tube with or without the MPC polymer coating was filled with a Tol 5 cell suspension and incubated for 10 min. Then, the cell suspension was replaced with fresh BS-N medium by slow flowing at 10 μL/min for rinsing, and the flow rate was increased stepwise, as shown in Figure 4B, while observing the inner surface of the bottom of the glass tube under a microscope. Unexpectedly, Tol 5 cells adhered to the MPC-polymer-coated glass as much as the bare (non-coated) glass under static conditions and remained adhered after rinsing at 10 μL/min (Fig 4C initial). When the flow rate was increased to 20 μL/min, a small fraction of previously adhered cell clumps started to move and slip on the surface (see Supplementary Movie S1), but many cells still resisted detachment after 10 min of flowing (Fig. 4C, 20 μL/min). At a high flow rate of 50 μL/min or more, the Tol 5 cells firmly adhered to the bare glass whereas the cells attached on the MPC polymer were exfoliated, rolled, and washed off from the surface by the shear stress (≥5.94 mN/m2) (Fig. 4C, ≥50 μL/min and see Supplementary Movie S1).

Discussion

So far, various antiadhesive materials have been developed on the basis of repelling mechanisms. Fluoropolymers with a low surface free energy are widely used in cookware and medical

equipment although their hydrophobicity is also said to cause protein adsorption that hinders cell attachment by contraries (*25*). Polymer brushes with a high grafting density have been especially studied as powerful antiadhesive surfaces for cell adhesion (*26*). However, the finding that *Acinetobacter* sp. Tol 5 is able to adhere to these antiadhesive materials makes us realize the marvel of microbial diversity and evolution. In addition, AtaA could mediate cell adhesion to poly(mOEGMA) brush but BadA could not. Note that BadA is similar to AtaA in size and abundance on the cell surface; it consists of 3,082 amino acids and its fibrous molecules peritrichately cover over bacterial cells (*18*). Therefore, their difference in adhesiveness demonstrates the functional diversity of the TAA family as a result of protein evolution.

Tol 5 cells even adhered to an MPC-polymer-coated surface but their interaction was so weak that the cells could be exfoliated by a weak shear stress. The exfoliated and rolling cell clumps seemed to involve and remove cell clumps that were still adhered owing to the autoagglutinating property of Tol 5 cells (*24*), self-cleaning the surface coated with the MPC polymer. In an adhesion assay using a microwell, the Tol 5 cells should have been detached by the washing step. MPC is a methacrylate monomer with a phosphorylcholine (PC) group, which is a hydrophilic polar head group of phospholipids comprising a eukaryotic cell membrane (*27*). MPC polymers are known to significantly suppress adhesion of proteins and cells because there are lots of free water molecules (*22*) but capture few bound water molecules on their PC group (*27-29*). The fact that Tol 5 cells can adhere to the poly(mOEGMA) brush and the mica, but can only interact very weakly with a surface coated with MPC polymers, despite similar levels of hydrophilicity, as shown by the static contact angles of air in water (Table S2), suggests that exchangeable bound water molecules contribute to the interaction between AtaA and material surfaces (*28*).

Materials and Methods

Materials

The polyurethane foam support (1 cm^3 cube; CFH-30) was obtained from Inoac Corporation (Aichi, Japan). The polystyrene plate (PS2035-1), glass plate (FF-001), stainless steel plate (SUS430 grade; EA441WA-21), PTFE plate (J1-537-01), mica disk (V-1 grade), and square glass tube (VitroTubes, 8100) were purchased from Hikari Co., ltd. (Osaka, Japan), Matsunami Glass Ind., Ltd. (Osaka, Japan), ESCO Co., Ltd. (Osaka, Japan), AS ONE Corp. (Osaka, Japan), TED PELLA, Inc. (CA, USA), and Vitrocom (Mountain Lakes, NJ), respectively. The poly(mOEGMA) brush surface was prepared as described previously (*29*). Prior to use, glass was washed with piranha solution followed by rinsing with pure water, mica was cleaved with scotch tape, and stainless steel and PTFE were rinsed with pure ethanol.

The MPC-polymer-coated surface for the adhesion assays was prepared as described below. A glass plate was dipped into 2% MPC polymer (Lipidure-CM5206, a copolymer of MPC and butyl methacrylate; NOF Corp., Tokyo, Japan) solution dissolved in ethanol and shaken at 70 rpm for 3 min. After shaking, the glass plate was rinsed in pure water and dried at 60°C for 3 h.

The MPC-polymer-coated glass tube was prepared as described below using the flow cell system. A solution of 2% MPC polymer dissolved in ethanol was flowed through the square glass tube (50 mm in length and 1 mm in every internal dimension) at 100 μL/min for 3 min, and then pure water was flowed through at 500 μL/min for 2 min. After passing air through at 500 μL/min for 2 min, the glass tube was removed from the flow cell system and dried at 60°C for 3 h.

The static contact angles of air bubbles (SCA) in water on each material surface were measured with a contact angle meter (CA-W, Kyowa Interface Science Co., Tokyo, Japan) at room temperature and listed in Supplementary Table S2. Material substrates were immersed in water, and 10 μL of air bubbles were placed on the substrates.

Microwells used in the adhesion assays to various material surfaces were self-made as follows: Four sheets of vinyl electrical tape (ASKUL Corporation, Tokyo, Japan) were piled up and holes

with a diameter of 6 mm were punched. The punched tapes were placed on a material plate as shown in Supplementary Figure S2.

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Supplementary Table S1. These bacterial strains were grown as previously described (*15, 30*). *Acinetobacter* strains were grown in Luria-Bertani (LB) medium at 28°C for 8 h. An overnight culture of *Yersinia enterocolitica* strains grown in LB medium at 28°C was used to inoculate LB medium at a 1:100 dilution, and the medium was incubated at 37°C for 6 h. *Bartonella henselae* strains were grown for 5 days on Columbia agar supplemented with 5% sheep blood at 37°C in a humidified atmosphere with 5% CO_2. Expression of trimeric autotransporter adhesins in each strain was confirmed by western blotting using anti-$AtaA_{699\text{-}1014}$ antiserum, anti-BadA antibody (*31*), and anti-YadA antibody (sc-22472; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Adhesion assays to polyurethane foam support

The adhesion assays to polyurethane foam support were performed as previously described (*32*), with slight modifications. Bacterial cells were suspended in BS-N medium (*33*) (containing no carbon or nitrogen sources), and the optical density of the cell suspension at 660 nm (OD_{660}) was adjusted to 1.0. Four pieces of the polyurethane foam support were placed into 20 mL of the cell suspension and shaken at 115 rpm at 28°C. After a 30-min incubation with shaking, the transparency of the cell suspension was observed and photographed by a digital camera.

Microwell adhesion assay

Bacterial cells were suspended in BS-N medium and the OD_{660} was adjusted to 0.5. The cell suspensions (50 μL each) were placed into the microwell on the materials and incubated at 28°C for 10 min. When the antiadhesive surfaces were used, the incubation time was extended to 30 min and 2 h. The cell suspensions were removed using a pipet after the incubation and the wells were washed for 10 s by shaking in BS-N medium at 70 rpm. Cells adhering in the well were stained with 50 μL of 0.1% crystal violet solution for 15 min and washed for 10 s by shaking in BS-N medium at 70 rpm. Finally, the stain was eluted with 200 μL of 70% ethanol, and the absorbance of the elution at 590 nm (A_{590}) was measured with a microplate reader (ARVO X3; PerkinElmer, Inc., MA, USA).

Flow cell system

The rectangular flow cell system, which we have previously reported (*24*), was used with slight modifications: a syringe pump (Legato 200; KD Scientific, Holliston, MA) was directly connected to the square glass tube without using a three-way stopcock valve. Tol 5 cells were suspended in BS-N medium at an OD_{660} of 0.2, and the suspension was subjected to sonication to break up the cell clumps. The glass tube with or without an MPC polymer coating was filled with the cell suspension and incubated for 10 min at room temperature. The suspension was replaced with fresh BS-N medium by slow flowing at 10 μL/min for 35 min and the flow rate was increased stepwise. Live images of the behavior of Tol 5 cells during their adhesion and detachment to/from the inner surface of the glass tube were recorded under a digital microscope (VHX-200; Keyence, Osaka, Japan). Wall shear stress was calculated as previously described (*24*).

H2: Supplementary Materials

Fig. S1. Production of trimeric autotransporter adhesin (TAA) in each bacterial cell.
Fig. S2. Preparation of microwells on material surfaces.

Table S1. Bacterial strains used in this study.
Table S2. Static contact angles (SCA) of air in water of material surfaces.
Movie S1. Detachment of adhered Tol 5 cells from the MPC polymer coated glass.

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Figures and Tables

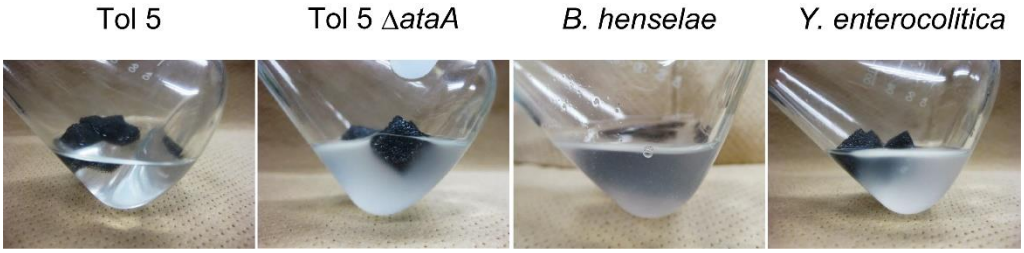

Fig. 1. Adhesion of bacterial cells to a polyurethane surface. Each panel shows the bacterial cell suspension after shaking for 30 min with a polyurethane foam support.

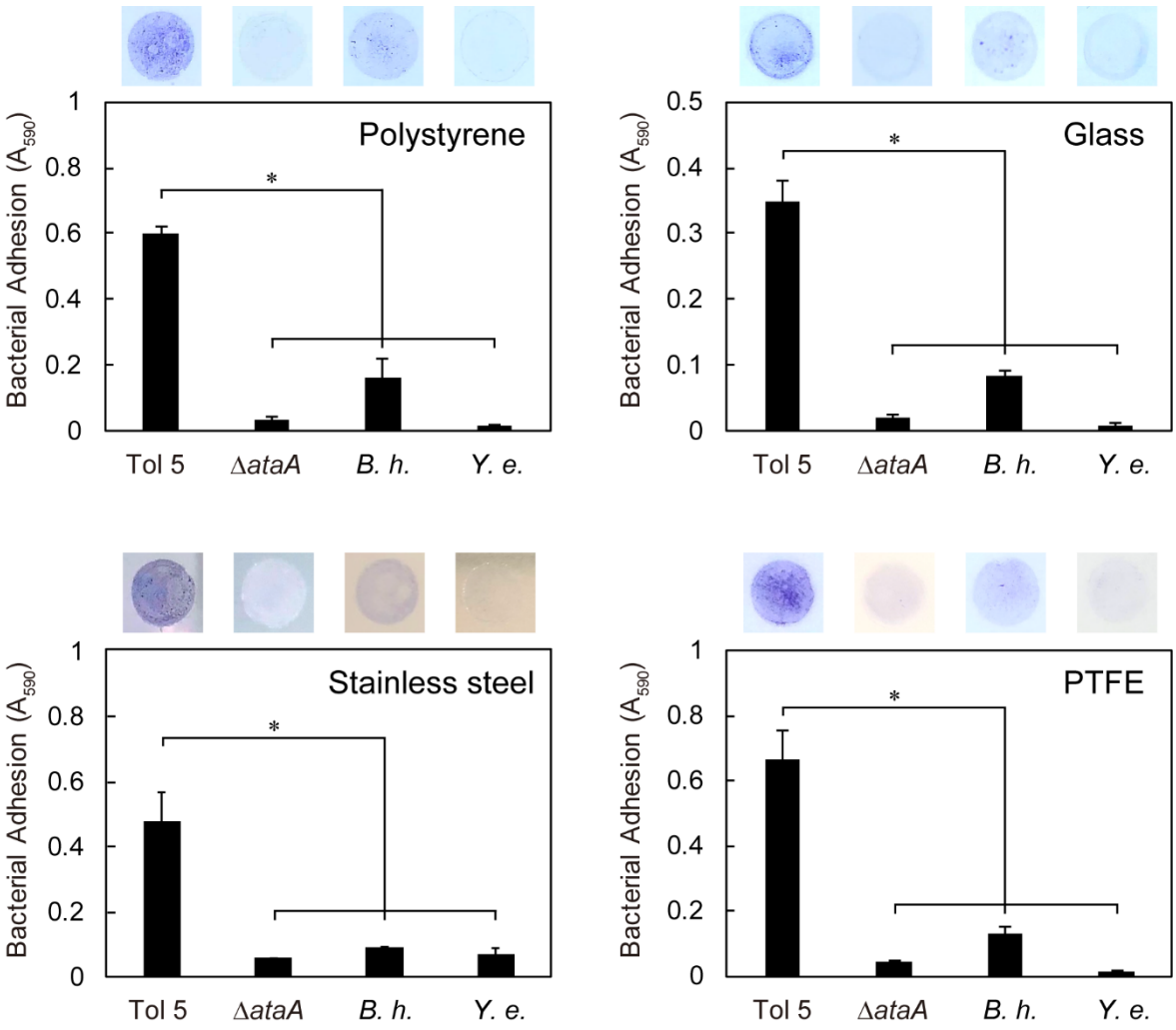

Fig. 2. Adhesion of bacterial cells to various materials. Adhesion of *Acinetobacter* sp. Tol 5, Tol 5 Δ*ataA*, *B. henselae* (*B. h.*), and *Y. enterocolitica* (*Y. e.*) to polystyrene, glass, stainless steel, and PTFE was assessed by microwell adhesion assays for 10 min. Data are expressed as the mean ± SEM (n=3). Significant differences from the result of Tol 5, analyzed using Student's t-test, are indicated by an asterisk ($p<0.05$). Upper photographs show the adhered cells on the material surfaces.

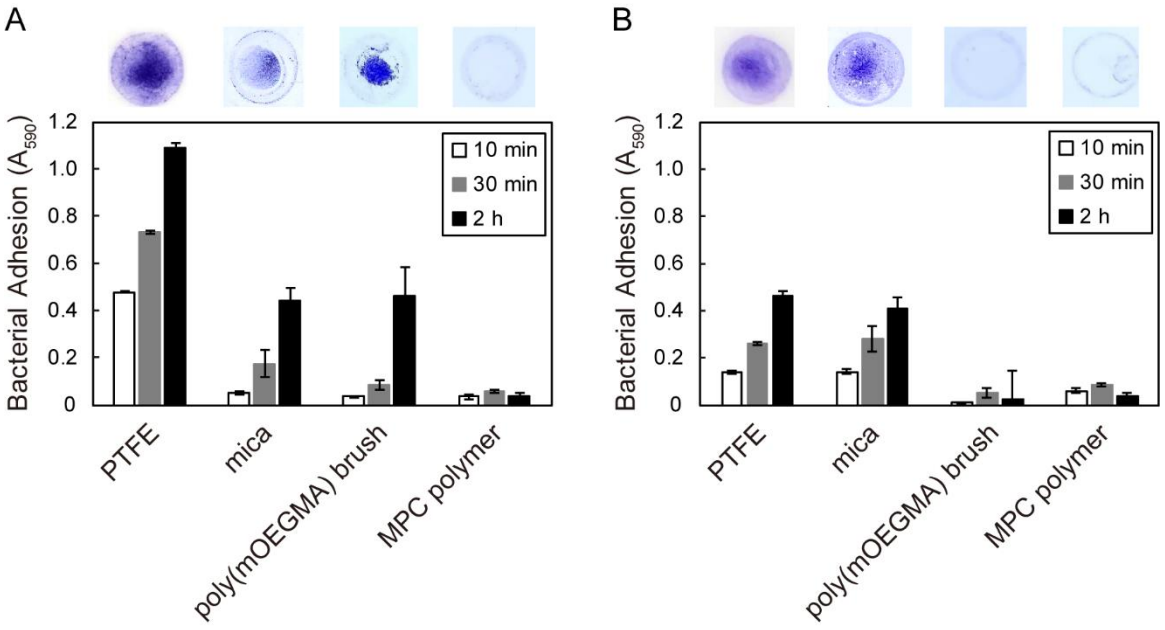

Fig. 3. Adhesion of bacterial cells to antiadhesive surfaces. Adhesion of Tol 5 (A) and *B. henselae* (B) to PTFE, mica, poly(mOEGMA) brush on glass, and MPC-polymer-coated glass, was assessed by microwell adhesion assays. Data are expressed as the mean ± SEM (n=3). Upper photographs show the adhered cells on the material surfaces after incubation for 2 h.

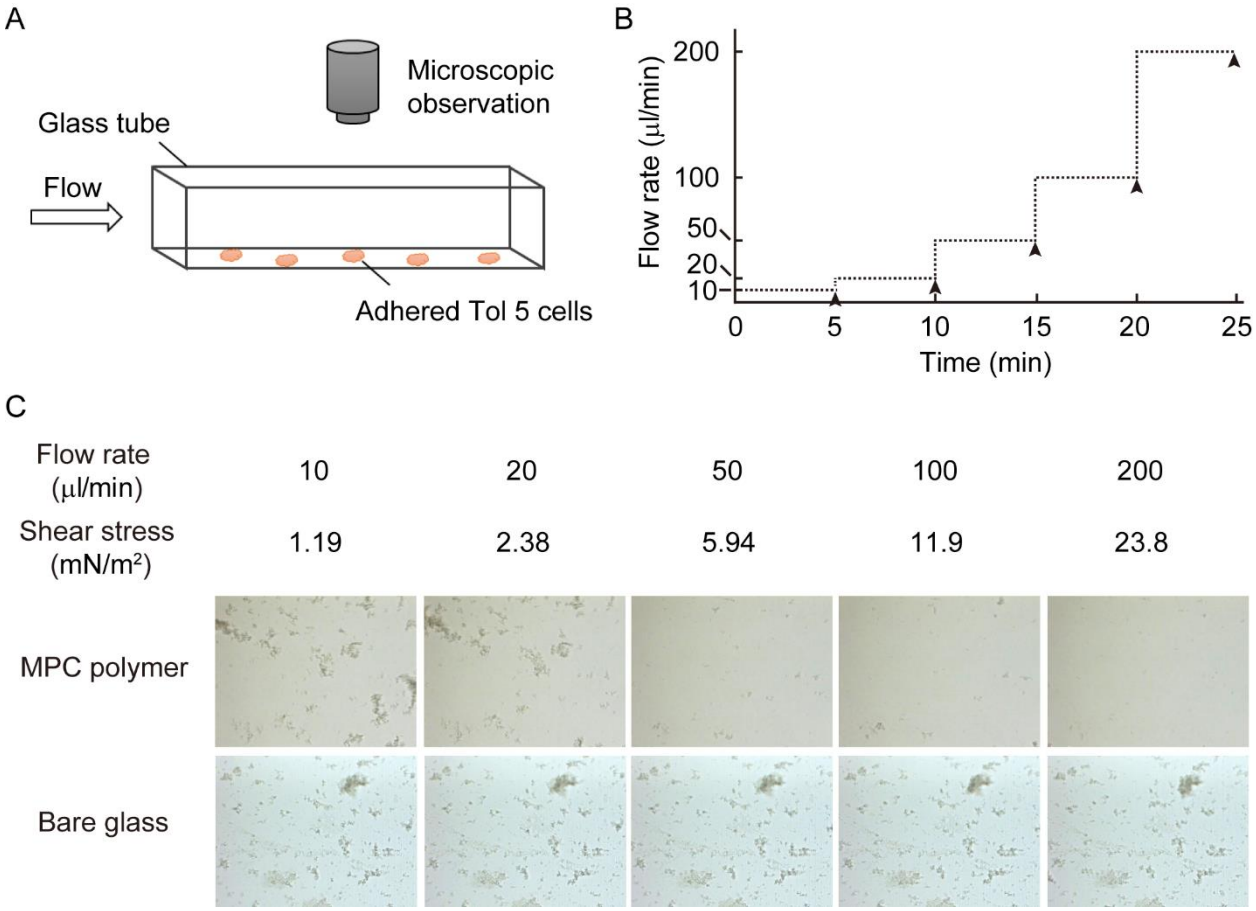

Fig. 4. Observation of the behavior of Tol 5 cells that were adhered to the MPC polymer surface beforehand. (A) Schematic representation of the flow cell system used in this study. (B) Transition of the flow rate. The flow rate was increased stepwise every 5 min. The black arrowheads indicate the time at which snapshots of the inner surface at the bottom of the glass tubes were captured. (C) The snapshots captured as described in (B).

Supplementary Materials

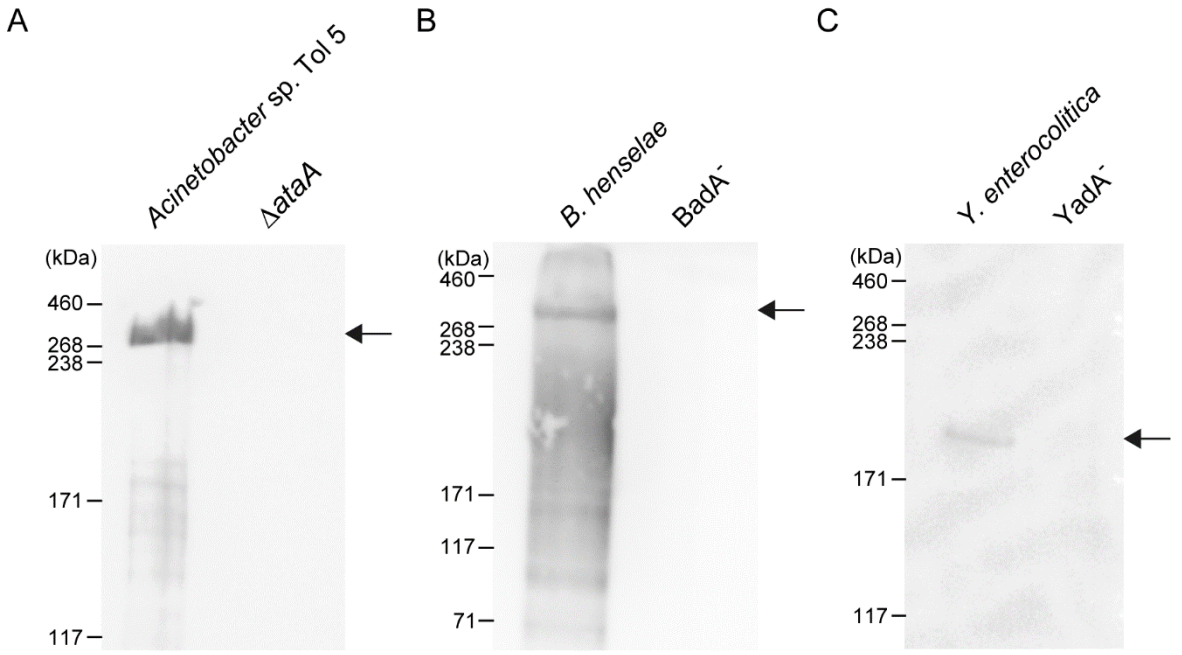

Fig. S1. Production of trimeric autotransporter adhesin (TAA) in each bacterial cell. Cell lysates of *Acinetobacter* sp. Tol 5 (A), *B. henselae* (B), and *Y. enterocolitica* (C) were separated by SDS-PAGE with their TAA-less mutant strains, and each TAA was detected by western blotting. The arrow in each panel indicates the band corresponding to AtaA monomer (A), BadA monomer (B), and YadA trimer (C).

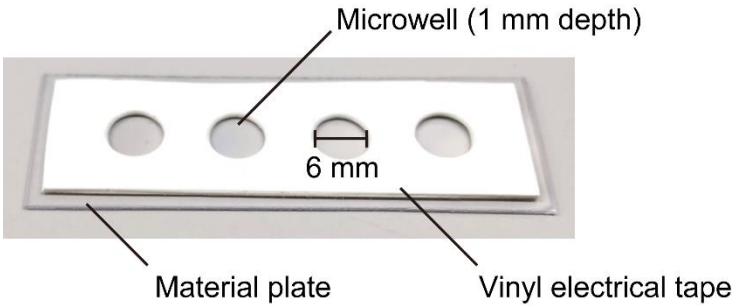

Fig. S2. Preparation of microwells on material surfaces. Four sheets of vinyl electrical tape were piled up and holes with a diameter of 6 mm were punched. The punched tapes were placed on a material plate.

Table S1. Bacterial strains used in this study.

Strain	Description	Reference
Acinetobacter sp. Tol 5	Wild type strain, expressing *ataA*	(*14*)
Acinetobacter sp. Tol 5 Δ*ataA*	*Acinetobacter* sp. Tol 5 4140, Unmarked Δ*ataA* mutant of Tol 5, *ataA*$^-$	(*34*)
Bartonella henselae	*B. henselae* Marseille, Patient isolate, expressing *badA*	(*35*)
Bartonella henselae BadA$^-$	*B. henselae* Marseille transposon mutant, transposon integrated in *badA*, Km^r	(*31*)
Yersinia enterocolitica	*Y. enterocolitica* WA-314 serotype O:8, harboring plasmid pYV, expressing *yadA*	(*36*)
Yersinia enterocolitica YadA$^-$	*Y. enterocolitica* WA-C, plasmid less derivative of WA-314, *yadA*$^-$	(*36*)

Table S2. Static contact angles (SCA) of air in water of material surfaces.

Material	Polystyrene	Glass	Stainless steel	PTFE	Mica	Poly(mOEGMA) brush	MPC polymer
SCA [deg]	99 ± 2	139 ± 3	101 ± 3	86 ± 4	142 ± 3	134 ± 2	154 ± 4

Data are expressed as the mean ± SD (n = 5).

Movie S1. Detachment of adhered Tol 5 cells from the MPC polymer coated glass. A glass tube with or without an MPC polymer coating was filled with Tol 5 cell suspension and incubated for 10 min. Then, the cell suspension was replaced with fresh BS-N medium by slow flowing at 10 μL/min for 35 min, and the flow rate was increased stepwise as shown in Figure 4B. Live images of the adhesion behavior of Tol 5 cells on the inner surface of the glass tubes were observed with a digital microscope.