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Doctor Thesis

**Analyses of cell adhesion properties of a toluene
degrading-bacterium, *Acinetobacter genospecies* Tol 5,
and its application to the biofiltration**

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Chapter I

Introduction

1-1. General introduction

There are two categories of bioprocesses to decrease toxic substances released through human civilization. One of them is a 'bioremediation' process, in which microorganisms degrade toxic compounds such as chlorinated organic compounds, spilled oil, excess nitrogen and phosphorus, and heavy metals. The toxic compounds have already contaminated the soil, atmosphere, and hydrosphere including underground water. The other one is a 'bioprevention' process, in which emissions to the natural environment released through wastewater or off-gas are prevented or reduced by using microbial conversion. The priority for environmental biotechnology is to reduce and prevent such damage by employing 'bioprevention'.

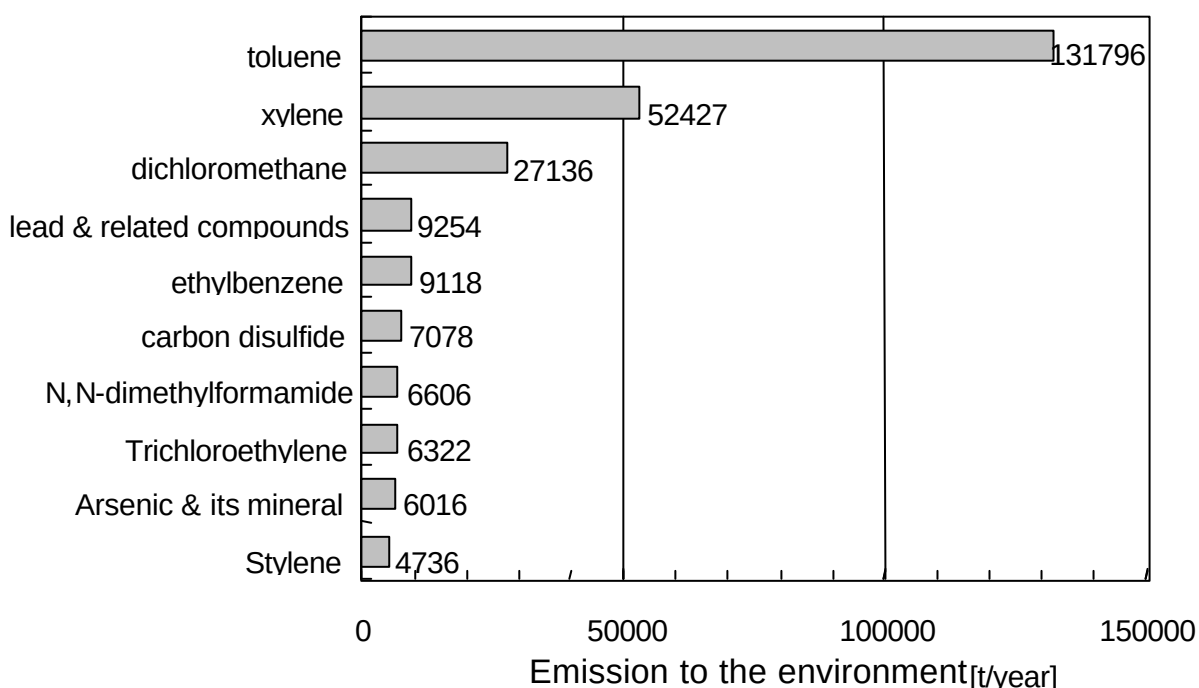


Fig. 1-1 Emission of the toxic substances to the environment in Japan from PRTR project of fiscal 2001 (2003.3 Ministry for the Environment)

Fig. 1-1 shows the emission of the toxic substances to the environment in Japan, which was reported in the PRTR project of fiscal year 2001. This report shows that toluene and xylene have been emitted to the environment extremely larger than the other toxic compounds. Hence, regulatory requirements against emissions of volatile organic carbons (VOC) have been urgent issue because of their high potential to damage ecological system.

A cost effective bioprevention process is biofiltration process, which has been used to decrease the emission of organic materials, ammonia, nitrate, and other toxic compounds from wastewater and off gas. The biofiltration process utilizes a microbial film fixed on support media where the contaminants are absorbed and converted to benign end products such as water and carbon dioxide [81]. Both the its degradation ability of toxic contaminant and bacterial adhesion ability to the support in the reactor are important to enhance the efficiency of this system.

Indeed, bacterial adhesion to the solid surface is beneficial to degrade toxic compounds when the specific bacterial degrader is used as a biocatalyst. The cell adhered to the support is considered as the fixed biocatalyst. For instance, Jun *et al.* suggested a new process on the wastewater treatment, in which a porous carrier and a membrane were added as the substratum and the filter for bacterial cells, respectively [33, 54]. In this system, the porous carrier facilitated nitrogen removal from wastewater by bacterial adhesion to them, resulting in the creation of an anaerobic region [96]. On the other hand, filtration by the membrane decreased the excess sludge-elimination, but the clogging of the membrane was caused by bacterial adhesion. Hence, both bacterial adhesion to the solid surface and desorption from them are important in that system. Correspondingly, for bioremediation process in the soil sites, microbial adhesion to the soil particles is also important. Control of microbial transport and retention in the soil is required for effective and safe bioremediation of contaminated soil and groundwater [8, 70].

In addition to degradation of environmental hazardous chemicals in soil [8, 44], bioreactors for wastewater [48] and off-gas treatment [29], bacterial adhesion processes can be also beneficial in agricultural uses of root nodule bacteria in the rhizosphere [17], in the degradation of biopolymers such as cellulose [69], and in biofloculants used for the separation of coal particles [68]. However, biofilm formation, which is the subsequent event of bacterial adhesion, has detrimental effects on various industrial processes. Biofilm, which consists of the multi-layer bacterial cluster, is problematic to both human life and industrial processes, causing infection associated with medical implants [5, 14], pathogen interaction with host cells [15, 23, 82], dental decay [67], contamination of food from processing equipments [40, 58], microbiologically influenced corrosion of metals [85], formation of marine biofilms on ships' hulls [12], and so on.

For those reasons, many researchers and engineers in various fields have studied the adhesive microorganisms. The research in recent years has emphasized obtaining information that suggests ways to prevent or control unwanted biofilm formation or biofouling. In the

biofiltration process, the regulation of adhesion is also important because bacterial degraders have to be fixed in the reactor and remove them when the reactor is clogged by excess biofilm.

1-2. Research background

Recently, Yamashita *et al.* performed the off-gases elimination using a trickle bed air biofilter (TBAB) made of urethane foam microbial carrier. In a TBAB, that is one of the biofiltration processes, inert synthetic support media are used for the filter bed. They demonstrated that the sufficient toluene elimination rate was obtained by this reactor for three months after acclimated period for 19 days, where the inoculum was activated sludge from an industrial wastewater treatment plant.

After three-month operation of the TBAB, sludge adhering to the support of packed bed was suspended in physiological saline. From them, five major strains which degraded toluene were isolated by repeating liquid culture and plating with supply of toluene as a sole carbon source. These strains were named as Tol 1 to Tol 5. The bacteria were identified by Gram staining, Micro Station System (BIOLOG) and 16S rRNA gene partial sequencing using MicroSeq™ Full Gene 16S rDNA Bacterial Sequencing Kit (PE Applied Biosystems). As a result, one of them, strain Tol 5 was a gram-negative bacillus and identified as *Acinetobacter genospecies 10* by using Micro Station System. The result of partial sequencing of 16S rRNA (Fig. 1-2) showed 96.4% similarity with *Acinetobacter* sp. DSM 590. The other four strains were gram-positive bacilli in the shape of short rod and identified as belonging to a genus *Rhodococcus* by Micro Station System and 16S rRNA partial sequences.

The isolates were grown on BS with toluene (100 µl-toluene/l-headspace), and toluene degradation was examined among the isolates. *A. genospecies* Tol 5 exhausted toluene in headspace within 2 d, while it took more than 3 d by the other four strains of *Rhodococcus*. The substrate range to be utilized as the sole carbon source was examined for *A. genospecies* Tol 5, *Rhodococcus* sp. Tol 1 and *Rhodococcus* sp. Tol 3. *A. genospecies* Tol 5 and *Rhodococcus* sp. Tol 3 utilized not only toluene but also other aromatics such as benzene, xylene. Therefore, these bacterial strains can be widely applied to elimination processes of benzene, toluene and xylene (BTX), which are typical components of petroleum. *A. genospecies* Tol 5 also grew well on ethanol, which is an inexpensive and easy-handling carbon source as a substrate for large culture of the strain. In these isolates, only *A. genospecies* Tol 5 had the strong adhesion property to the support media. Consequently, this strain has a possibility to play an important role to degrade the toluene in the TBAB.

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1 tagagtttga tcatggctca gattgaacgc tggcggcagg cttaacacat gcaagtcgag
61 cgggggawgt tgcttcggta actgacctag cggcggacgg ttgagtaata cttaggaatc
121 tgcctattaa tgggggacaa catctcgaaa gggatgctaa taccgcatac gccctacggg
181 ggaaagcagg ggatcacttg tgaccttgcg ttaatagatg agcctaagtc ggattagcta
241 gttggtgggg taaaggccta ccaaggcgac gatctgtagc gggctctgaga ggatgatccg
301 ccacactggg actgagacac ggcccagact cctacgggag gcagcagtg ggaatattgg
361 acaatggggg gaaccctgat ccagccatgc cgcgtgtgtg aagaaggcct tatggttgta
421 aagcacttta agcgaggagg aggctctttt ggttaatacc caagatgagt ggacgttact
481 cgcagaataa gcaccggcta actctgtgcc agcagccgcg gtaatacaga gggtgcgagc
541 gttaatcgga tttactgggc gtaaagcgtg cgtaggcggc tttttaagtc ggatgtgaaa
601 tccccgagct taacttggga attgcattcg atactgggaa gctagagtat gggagaggat
661 ggtagaattc caggtgtagc ggtgaaatgc gtagagatct ggaggaatac cgatggcgaa
721 ggcagccatc tggcctaata ctgacgctga ggtacgaaag catggggagc aaacaggatt
781 agataccctg gtagtccatg ccgtaaacga tgtctactag ccgttggggc ctttgagact
841 ttagtggcgc agctaacgcg ataagtagac cgcctgggga gtacggtcgc aagactaaaa
901 ctcaaataa ttgacggggg cccgcacaag cgggtggagca tgtggtttaa ttcgatgcaa
961 cgcgaagaac cttacctggt cttgacatag taagaacttt ccagagatgg attggtgcct
1021 tcgggaactt acatacaggt gctgcatggc tgctgctcagc tcgtgtcgtg agatgttggg
1081 ttaagtcccg caacgagcgc aacccttttc cttacttgcc agcatttcgg atgggaactt
1141 taaggatact gccagtgaca aactggagga aggcggggac gacgtcaagt catcatggcc
1201 cttacgacca gggctacaca cgtgctacaa tggtcggtag aaagggttgc tacctagcga
1261 taggatgcta atctcaaaaa gccgatcgta gtccggattg gagtctgcaa ctcgactcca
1321 tgaagtcgga atcgctagta atcgcgatc agaatgccgc ggtgaatacg ttcccgggcc
1381 ttgtacacac cgcccgtcac accatgggag tttgttgac cagaagtagg tagtctaacc
1441 gtaaggagga cgcttaccac ggtgtggccg atgactgggg tgaagtcgta acaaggtaac

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Fig. 1-2 The partial sequence of 16S rRNA extracted from strain Tol 5,
which has toluene-degrading property

1-3. Objective of research

A toluene-degrading bacterium, *Acinetobacter genospecies* Tol 5, shows high adhesiveness to solid surfaces. The objectives of this research are as follows:

- Application of the adhesive bacterium, *A. genospecies* Tol 5, to a biofiltration system
- To reveal cell surface structures involved in tenacious adhesion of *A. genospecies* Tol 5
- Elucidation of the mechanism underlying cell adhesion of *A. genospecies* Tol 5

1-4. Outline of this research

Fig. 1-3 shows the overall research scheme of this dissertation. In chapter II, the characteristics of toluene degradation and carrier adherence of *A. genospecies* Tol 5 were analyzed. Then the suitability to apply this strain for an inoculum source of TBAB was explored. In chapter III, it was shown that the notable adhesion property of Tol 5 was responsible for the balloon like interaction through the novel cell surface appendage. The appendages were named straight anchor and the peritrichate pili-like appendage. In chapter IV, it was estimated that the novel adhesion mechanism was functioned through the anchor-like appendages in Tol 5. The one-step adhesion mechanism from the long distance through the anchor-like appendage was proposed for the adhesion dynamics of strongly adhesive Tol 5.

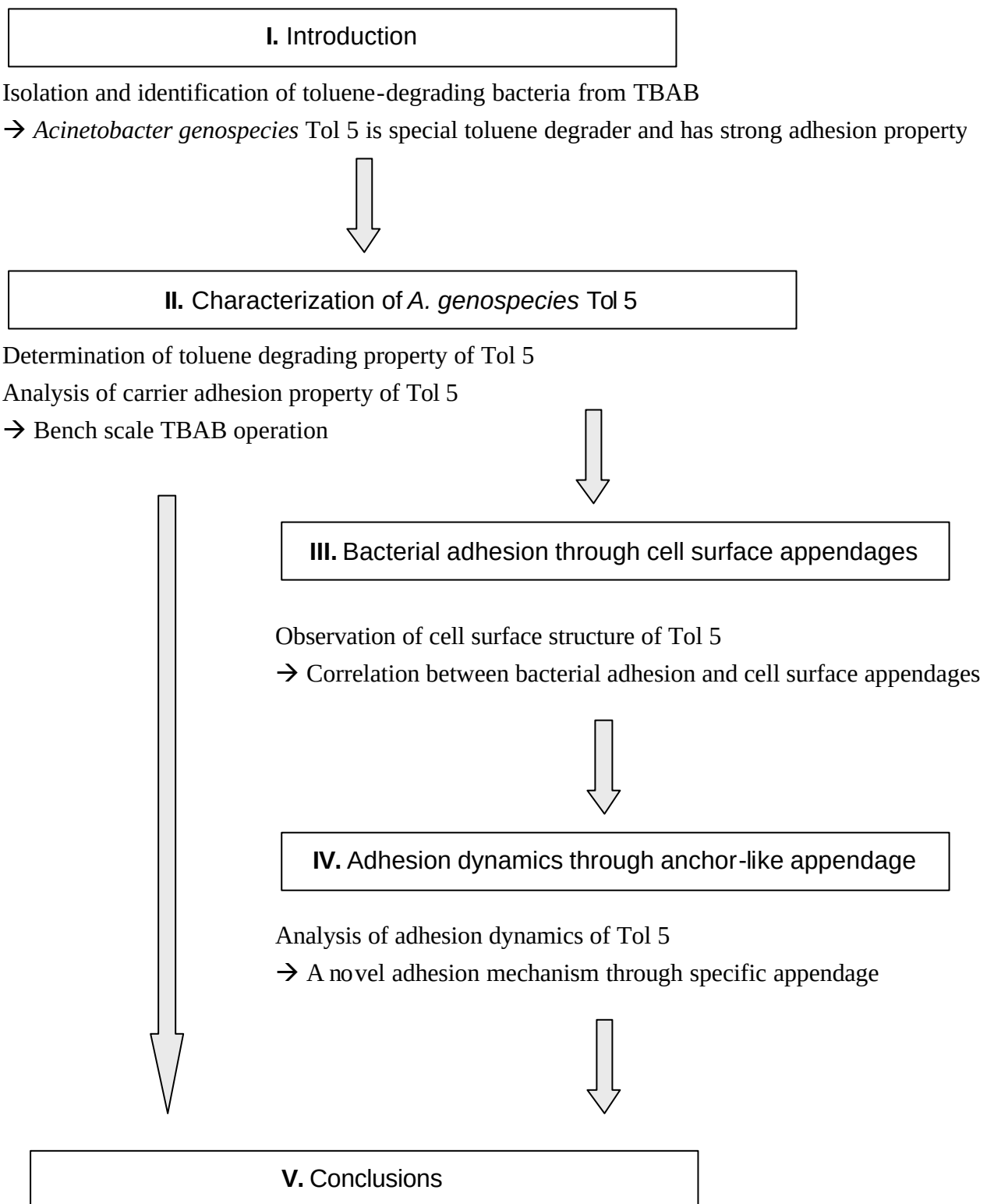


Fig. 1-3 Overview of this thesis

Chapter II

Characterization of *A. genospecies* Tol 5

2-1. Introduction

Regulatory requirements against emissions of volatile organic carbons (VOC) have been getting strict more and more because of their negative effect for ecological system. Especially, toluene has been exhausted to the environment via off-gas (Fig. 1-1). The physical adsorption process and the chemical incineration process have been applied to treat the elimination of the VOC in the off-gas. The bioprocess like biodegradation has generated a new demand for cost-effective technologies to control their emissions. The biofiltration process utilizes a microbial film fixed on support media in a single reactor where the contaminants are absorbed from the off-gas and converted to benign end products such as water and carbon dioxide [81] (Fig. 2-1). Due to lower operating costs, biofiltration can provide significant economic advantages over other air pollutant control technologies [42].

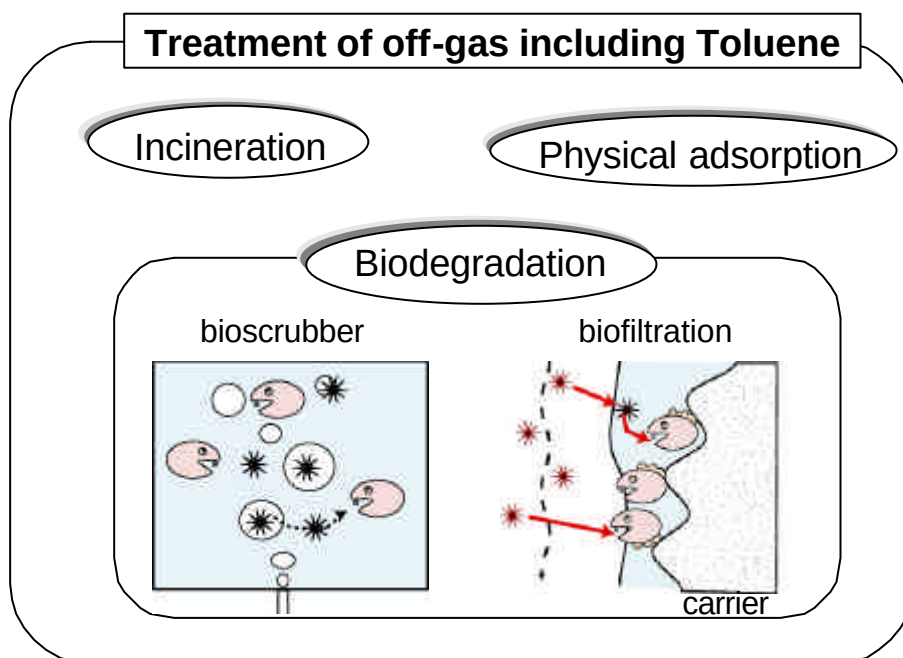


Fig. 2-1 Typical processes to treat the off-gas with VOC

In a trickle bed air biofilter (TBAB) inert synthetic support media are used for the filter bed [81]. A TBAB requires an inoculation with an appropriate microbial culture. Although activated sludge can be used as such a source, it does not have sufficient capability for elimination of VOC at the beginning of operation. Therefore, a long adaptation period (2-4 weeks) is required for acclimation of bacteria [34, 35]. Addition of a specific bacterial degrader to the filter can shorten the adaptation period at the starting up or restart of the process [42]. In order to apply specific bacteria to a TBAB, not only degradation ability but also easiness of their handling should be considered. For example, adhesion property of bacteria to the support packed in a reactor affects operability of treatment-facilities.

In this chapter, toluene-degrading properties of *Acinetobacter genospecies* Tol 5, which was isolated from the TBAB, were analyzed. Then its ability of adhesion to the polyurethane support was analyzed. Furthermore, the effectiveness of inoculation of the toluene-degrading bacterium for shortening the adaptation period was examined.

2-2. Materials and methods

2-2-1. Bacterial strain and culture

Toluene-degrading bacteria, *A. genospecies* Tol 5, *Rhodococcus* sp. Tol 2, and *Rhodococcus* sp. Tol 3, were isolated from sludge acclimated to toluene in an off-gas treatment reactor. The bacterial stains were grown on 20 ml BS medium (Table 2-1) in 125 ml vials. Each vial was added by 10 μ l of toluene (equilibrated aqueous toluene concentration was 164 μ g/ml) and sealed with a butyl rubber stopper and an aluminum cap. The vials were shaken at 70 rpm under 28°C.

Table 2-1 Composition of BS medium and ME solution

A. BS medium		B. ME solution	
<u>Component</u>	<u>concentration [g/l]</u>	<u>Component</u>	<u>concentration [g/l]</u>
Na ₂ HPO ₄	4.9	FeSO ₄ •7H ₂ O	2.8
KH ₂ PO ₄	2.0	MnSO ₄ •5H ₂ O	2.4
(NH ₄) ₂ SO ₄	2.0	CoCl ₂ •6H ₂ O	2.4
MgCl ₂ •6H ₂ O	0.34	CaCl ₂ •2H ₂ O	1.7
<u>ME solution</u>	<u>1 ml</u>	CuCl ₂ •2H ₂ O	0.2
	pH 7.2	ZnSO ₄ •7H ₂ O	0.3
		NaMoO ₄	0.25
		<u>conc. HCl</u>	<u>80 ml</u>

2-2-2. Analytical method

The concentration of toluene was measured by using a gas chromatography system (GC) equipped with a flame ionization detector (FID) (GC-17A, Shimadzu, Kyoto, Japan). The column used was ULBON HR-1 (I.D., 0.25 mm; length, 50 m; thickness of film, 0.25 μm , Shinwa Chemical Industries, Japan). Gas sample was taken from the headspace by using a gas tight syringe, followed by being injected into GC. In order to determine total amount of toluene in a vial and the liquid concentration of toluene, it was assumed that Henry's law was held under the experimental condition. Total amount of toluene in a vial was evaluated from a calibration curve of the headspace concentration of toluene vs. amount of toluene initially added. Henry's constant for toluene in BS at 28°C was determined from the headspace concentrations analyzed and the liquid concentrations calculated from the amounts of toluene initially and the headspace concentrations.

Biomass was analyzed by weighing dried cells. After cultivation, cells were harvested by centrifugation, washed with phosphate buffered saline (PBS) and lyophilized to get the dry cells. The number of toluene-degrading bacteria was analyzed by counting colony-forming-unit (CFU) on a BS agar plate in a desiccator supplied with toluene vapor.

2-2-3. Adhesion test of bacterial cells to a support material

The support used was a rectangular prism piece of polyurethane foam (1.25 cm $\times$ 1.25 cm $\times$ 1 cm), with the specific surface area, density and porosity of 12 cm²/cm³, 115 mg/cm³, and 73.8%, respectively. The support was added to 100 ml of BS in a 500 ml Erlenmeyer flask. A bacterium was inoculated followed by addition of toluene. The amount of the support was changed so that the ratio of total dry cell weight to the weight of the support would be varied after growth. The flask was capped with a rubber stopper and shaken at 120 rpm. After cultivation at 28°C, dry weights of cells adhering to the support and of cells in suspension were measured.

2-2-4. Measurement of cell surface hydrophobicity and z potential

Cell surface hydrophobicity was analyzed by contact angle measurements and microbial-adhesion-to-hydrocarbons (MATH) test.

Measurements of a contact angle

bacterial strains were grown on BS with toluene or Luria-Bertani (LB) medium, harvested by centrifugation, and resuspended in PBS. Thereafter, the cell suspensions were filtrated to get bacterial mats on polycarbonate filters (pore size, 0.2 μm ; diameter, 47 mm). The bacterial mats were dried for two hours at a room temperature. One drop of water was placed on a surface of the bacterial mat and its contact angle was measured (Kyowa Interface Science, Tokyo, Japan) (Fig. 2-2).

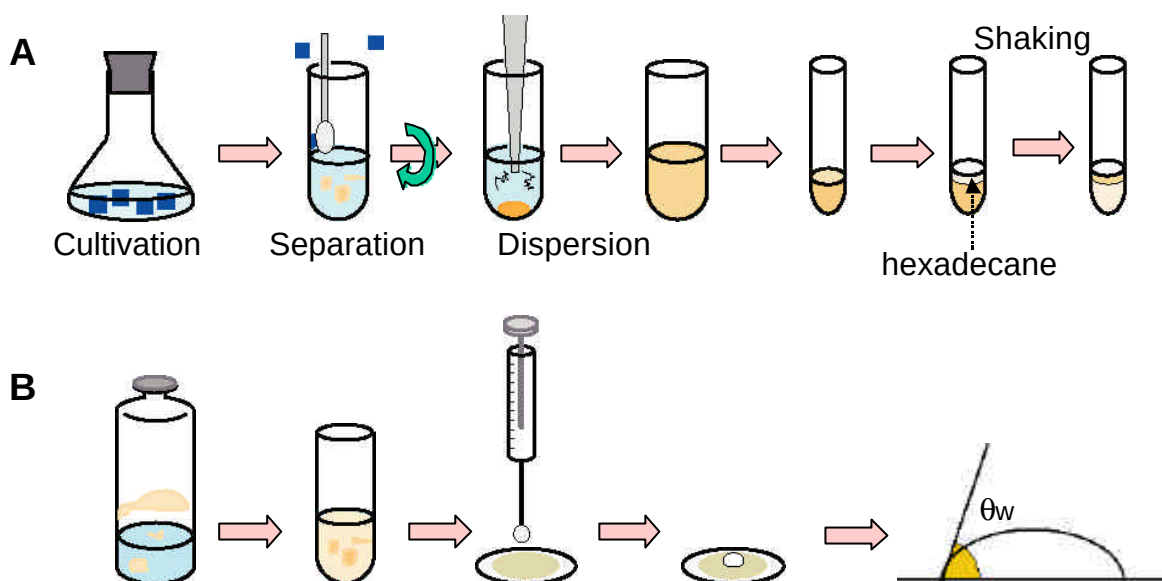


Fig. 2-2 Analytical methods for determination of cell surface hydrophobicity.

A: Microbial-adhesion-to-hydrocarbons (MATH) test was determined by the adhesion ratio to the hexadecane droplets. **B:** One drop of water was placed on a surface of the bacterial mat and its contact angle was measured.

MATH test

Cells were harvested by centrifugation (4000 rpm, 10 min), washed with sterile water and resuspended in BS. Optical density (OD_{660}) of the cell suspension was measured. To 1.0 ml of the cell suspension, 200 μ l 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 (Fig. 2-2).

$$\text{MATH (\%)} = \frac{OD_{660\text{-before}} - OD_{660\text{-after treatment}}}{OD_{660\text{-before}}} \times 100$$

z potential measurement

In stationary phase, cells were harvested by centrifugation ($1600 \times g$, 10 min), resuspended in 9.4 mM PBS (NaCl, 0.493 g; KH_2PO_4 , 29 mg; K_2HPO_4 , 0.119 g; per liter) (pH, 7.4, ionic strength, 13.9 mM). Cells were diluted to 40 μ g-dry cell/l. ζ potential was measured at 25°C by ELS-800 (Otsuka electronics co.,Ltd. Japan).

2-2-5. Operation of the TBAB

Bench scale experiment was demonstrated by using a TBAB system with polyurethane foam microbial carrier where *A. genospecies* Tol 5 was isolated (Fig. 2-3). The reactor was a packed column type (68 mm in inner diameter and 1.2 m in length), which equipped with a water sprinkler and a water circulation line with a reservoir tank (6 L), the bed volume being 1.63 L. The packed bed space was kept at 28°C using a water jacket. BS medium (Table 2-1) in the reservoir was sprinkled (flow rate: 9.6 L/h) to provide bacteria with water and nutrients. Model off-gas was prepared by dilution of toluene vapor, which had been purged from the liquid reagent by nitrogen gas, with air supplied from a compressor so as to be 100 μL -toluene gas/L-total gas. Mass flow controllers (MFC) were used to get the precise concentration of toluene in the model off-gas.

The support attached by the *A. genospecies* Tol 5 was packed into the column. Initially, concentration of the cells was 2.3 mg-cell/cm³-packing space. The model off-gas was fed to the column at 174 L/h, residence time of gas in the packed column being 0.57 min.

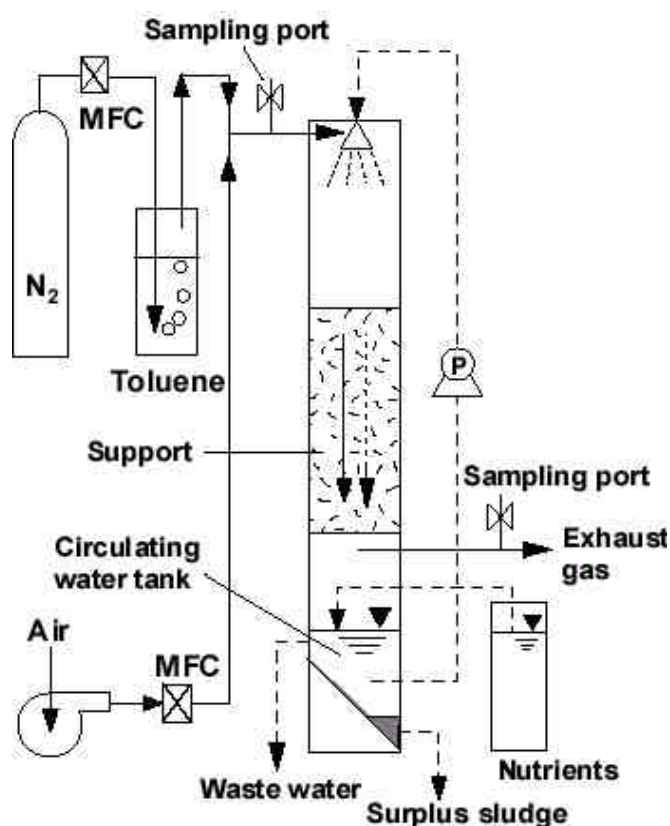


Fig. 2-3 Schematic diagram of the trickle bed air biofilter used for toluene elimination. Arrows with a solid line and with a broken line display a gas flow and a liquid flow, respectively. MFC and P display a mass flow controller and a liquid pump, respectively.

2-3. Results and discussion

2-3-1. Kinetics of growth and toluene degradation of *A. genospecies* Tol 5

Fig. 2-4 shows toluene degradation and growth curve of *A. genospecies* Tol 5 grown on toluene as the sole carbon source. Toluene added to the vial (10 μ l in 125 ml of the vial) was completely degraded within 37 h. The maximum specific growth rate ($\mu_{\max}$), doubling time (t_d) and growth yield ($Y_{X/S}$) were calculated to be 0.22 h^{-1} , 3.2 h and 0.37 g-cell/g-toluene, respectively.

In order to obtain kinetic data, toluene degradation experiments were conducted using resting cells of *A. genospecies* Tol 5. The cells harvested from the preculture and toluene were added to BS deprived of nitrogen source in vials so as to analyze initial degradation rates independently of cell growth. Dimensionless Henry's constant for toluene in BS at 28°C was experimentally determined to be 0.31, showing the same value in pure water at 30°C as reported in a literature [62]. The value was used to deduce the concentration of toluene in the medium. Fig. 2-5 gives the relationship between the concentration of toluene in the medium and the rate of toluene removal (A), and its double-reciprocal plot (B). In Fig. 2-5, substrate inhibition is recognized over 0.025 g/l of toluene in the medium. The maximum toluene removal rate by the bacterium, $V_{\max}$, is determined to be 6.5 mg-toluene/(g-cell · min) from Fig. 2-5 (A), even though, without the substrate inhibition, $V_{\max}$ would be deduced to be 12 mg-toluene/(g-cell · min) from an approximated line for the experimental data set except for deviating data from the line in Fig. 2-5 (B). The saturation constant of toluene, K_s , was determined to be 0.017 g/l from the

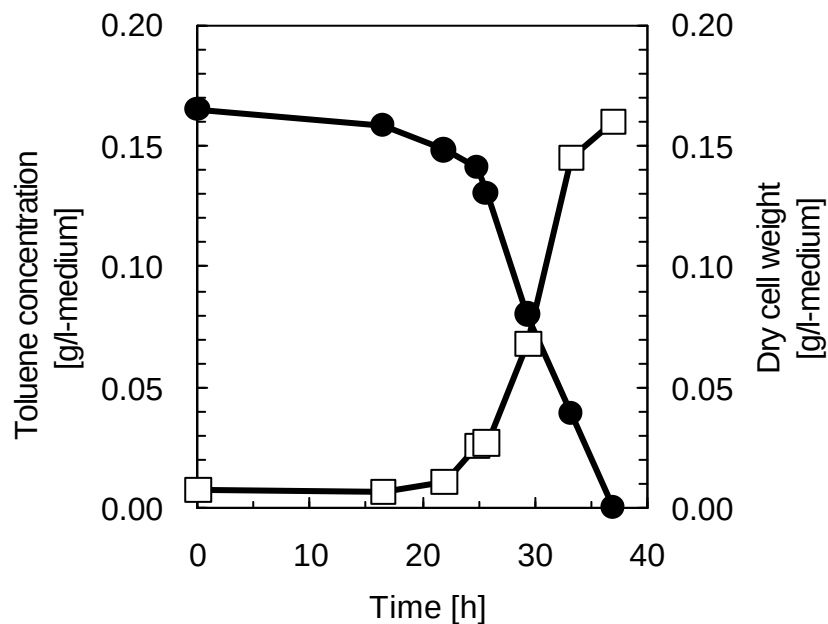


Fig. 2-4 Growth of *A. genospecies* Tol 5 on toluene and degradation of toluene. Open square: Dry cell weight. Solid circle: toluene degradation

approximated line. Similar experiments were also conducted using the original sludge from which *A. genospecies* Tol 5 had been isolated. As a result, the maximum toluene removal rate by the original sludge was 0.7 mg-toluene/(g-sludge · min), which was about one-tenth of that by *A. genospecies* Tol 5. This result suggests that *A. genospecies* Tol 5 dominantly contributed to the toluene elimination in the TBAB.

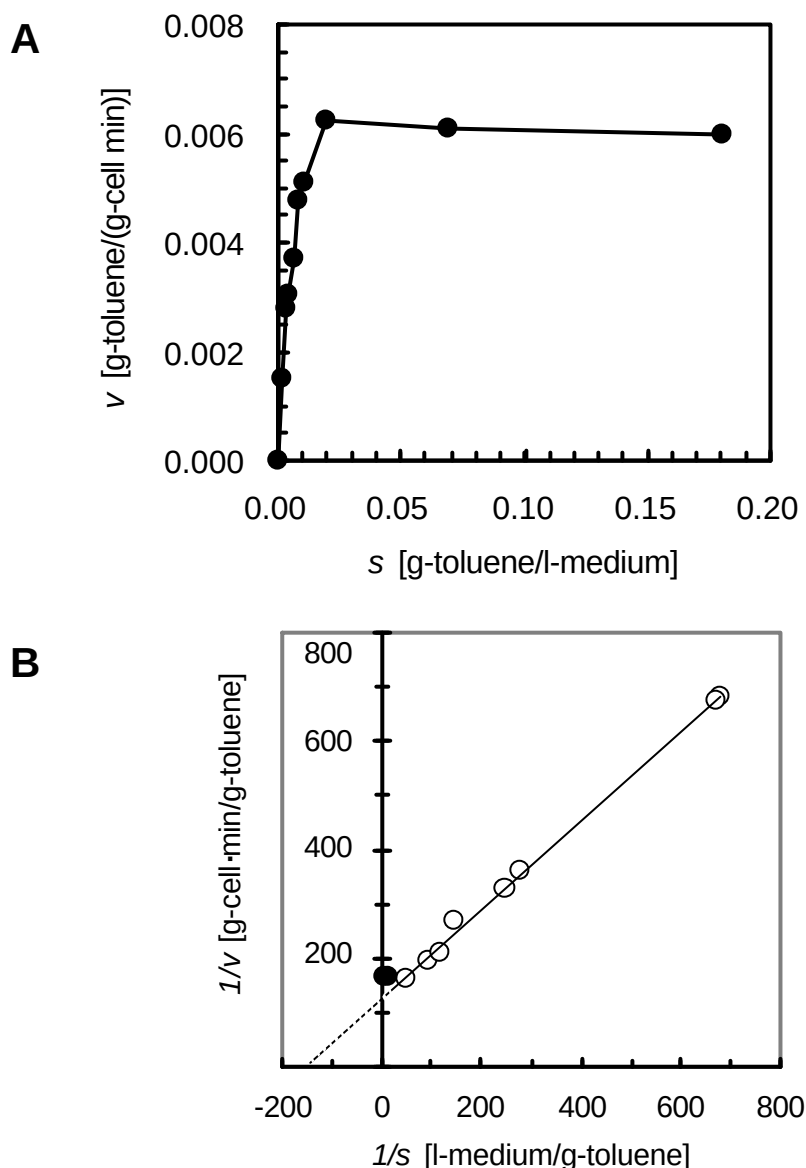


Fig. 2-5 Relationship between the concentration of toluene in the medium, s (g-toluene/l-medium), and the initial toluene-removal rate, v (g-toluene/(g-cell min)), **A** and its double-reciprocal plot **B**. **B**: a solid line is obtained from approximation for the experimental data set (open circles) except for data (solid circles) on which substrate inhibition was observed

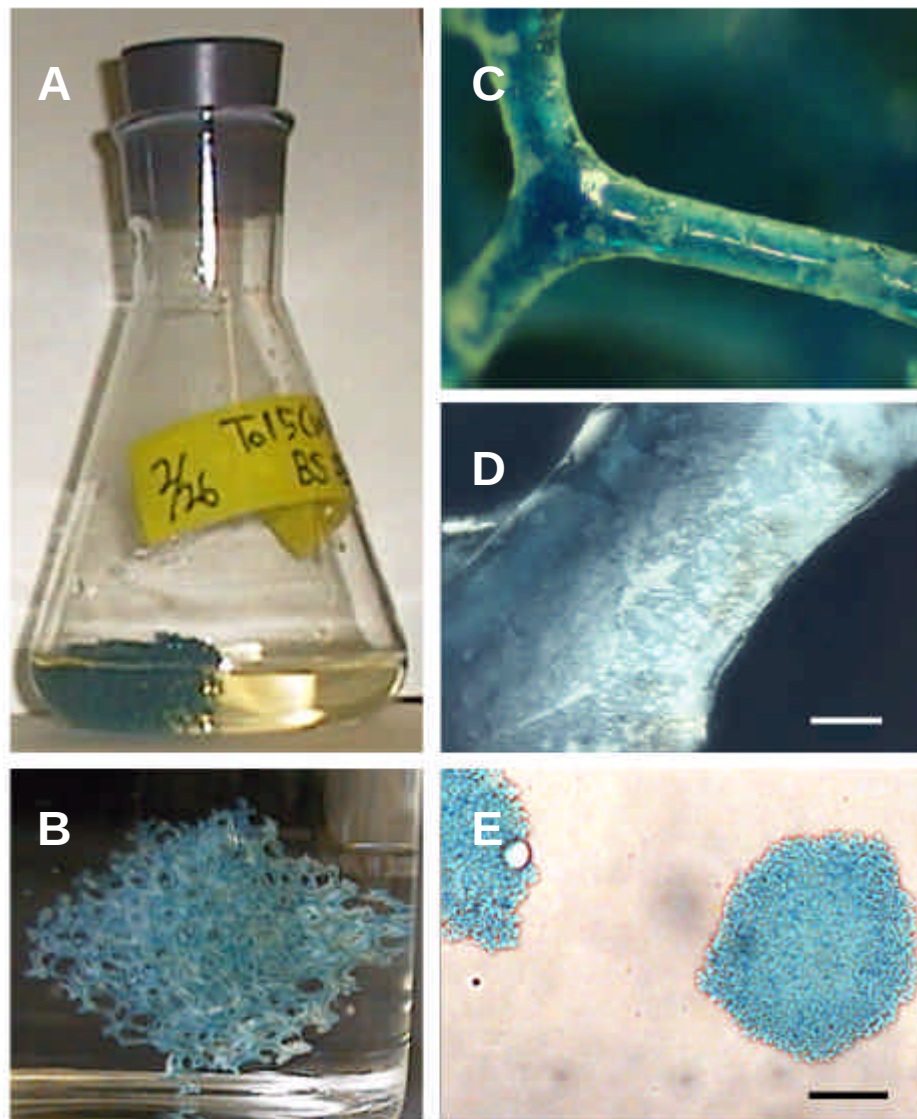


Fig. 2-6 Adhesion to the polyurethane foam of *A. genospecies* Tol 5.
A: Full growth culture on BS medium in the 100 ml Erlenmeyer flask with polyurethane carrier. **B:** Bacterial cells adhered to the polyurethane carrier. **C:** Close-up image of polyurethane carrier observed by stereoscopic microscope. **D:** Polyurethane surface to which the bacterial cells adhere observed by a confocal laser microscope. Bar = 100 μm . **E:** Bacterial cell aggregates separated from the polyurethane carrier by vigorous shaking. Bar = 25 μm .

2-3-2. Adhesion ability of *A. genospecies* Tol 5 to the solid surface

The cell of *A. genospecies* Tol 5 tended to adhere to glassware or polyurethane foam. *A. genospecies* Tol 5 was grown on BS with toluene in the presence of the polyurethane foam using a 100 ml or 500 ml Erlenmeyer flask. The polyurethane carrier with the adhered bacterial cells was observed by using microscopes (Fig. 2-6). Almost all of bacterial cells were adhered to the carrier, resulting in the culture medium was clear (Fig. 2-6 A). Highly-magnified image showed that bacterial cells adhered all over the carrier surface (Fig. 2-6 D). Many bacterial cells adhered to the carrier in the form of large cell aggregates, hence, it is suggested that bacterial cells adhere to the carrier at the multi-layer (Fig. 2-6 E).

After reaching the stationary phase of growth, the amount of cells adhering to the polyurethane foam was weighed together with that of cells in suspension. As shown in Fig. 2-7, almost all cells adhered to the polyurethane foam up to 0.12 g-cell/g-polyurethane. The maximum adherent amount was about 0.14 g-cell/g-polyurethane. On the other hand, the other isolated strains hardly adhered to the polyurethane foam (data not shown). The high ability of *A. genospecies* Tol 5 in adhesion to the support material is advantageous for its high concentration culture in a TBAB by withstanding against washout.

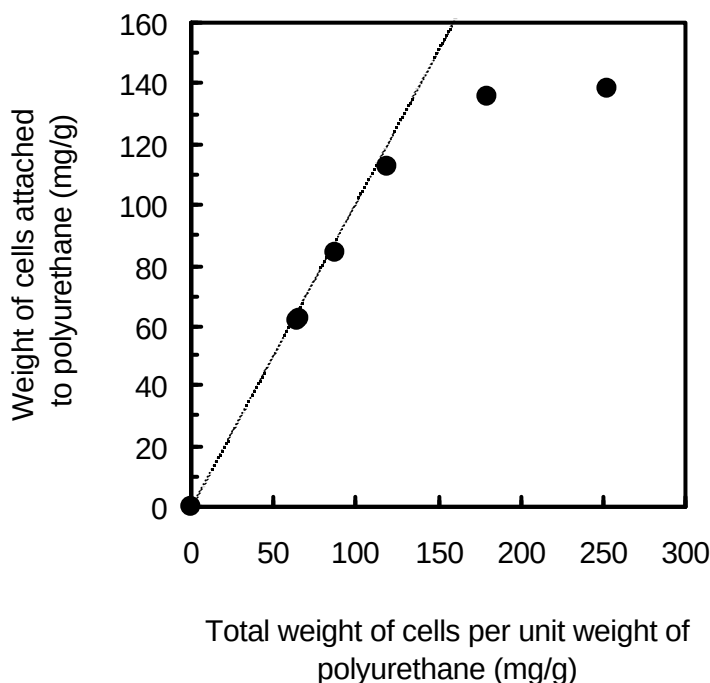


Fig. 2-7 Adherent amount of the bacterial cells to polyurethane foam. Symbols display experimental data set. A broken line displays the line at which all of cells adhere to polyurethane foam

2-3-3. Cell surface hydrophobicity and z potential

Immobilized cells of *A. genospecies* Tol 5 had a characteristic of water-repellent, suggesting a highly hydrophobic cell surface. Therefore, cell surface hydrophobicity of the strain was analyzed by measuring a contact angle and MATH test. As Table 2-2 shows, *A. genospecies* Tol 5 exhibited the quite high value in MATH test compared with the other isolates or *E. coli*. The contact angle against a water-droplet (θ_w) of *A. genospecies* Tol 5 was also the highest value among those of bacterial strains analyzed. There was a classification that strains with θ_w values of <30 to 35° are considered hydrophilic and those with θ_w values of $>70^\circ$ are considered hydrophobic (Bastiaens *et al.* 2000). Therefore, *A. genospecies* Tol 5 and *Rhodococcus* sp. Tol 3 are classified into hydrophobic strains. The extremely hydrophobic cell surface of *A. genospecies* Tol5 might have taken an important role in uptake of hydrophobic substrates like toluene and adhering to hydrophobic support like polyurethane.

The value of ζ potential was proposed as the criterion of cell surface hydrophobicity [43, 53, 87]. Moreover, ζ potential is an important parameter to elucidate the electrostatic interaction between the cells and a substratum [22, 53, 64, 89]. The absolute value of *A. genospecies* Tol 5 was quite low among those of bacterial strains analyzed. This was consistent with the result of determination of cell surface hydrophobicity.

Table 2-2 Analysis of cell surface hydrophobicity and ζ potential of bacteria

Bacterial strain	MATH test (%)*	Contact angle ($^\circ$)	ζ potential (mV) [†]
<i>A. genospecies</i> Tol 5	92	93	– 13.2
<i>Rhodococcus</i> sp. Tol 2	4.5	63	– 45.5
<i>Rhodococcus</i> sp. Tol 3	10	72	– 46.1
<i>E. coli</i> JM109	4.1	38	– 35.5

* Hydrophobicity (%) = $(OD_{660\text{-initial}} - OD_{660\text{-after extraction}})/OD_{660\text{-initial}} \times 100$

[†] ζ potential were measured in 9.4 mM PBS (I = 0.014 M) at pH 7.4.

2-3-4. Effectiveness of inoculation of *A. genospecies Tol 5* on operation of a TBAB

Yamashita *et al.* conducted the TBAB after preliminary operation for 7 d to get growth and immobilization to the support media of the inoculated activated sludge. Fig. 2-8 showed the performance for toluene elimination by the reactor for four-month operation. Although the reactor demonstrated the excellent performance for toluene elimination, it took about 20 d for the elimination to reach a sufficient level (an adaptation period). As Fig. 2-8 shows, the elimination rate and removal ratio markedly increased from 12 d to 19 d of the operation.

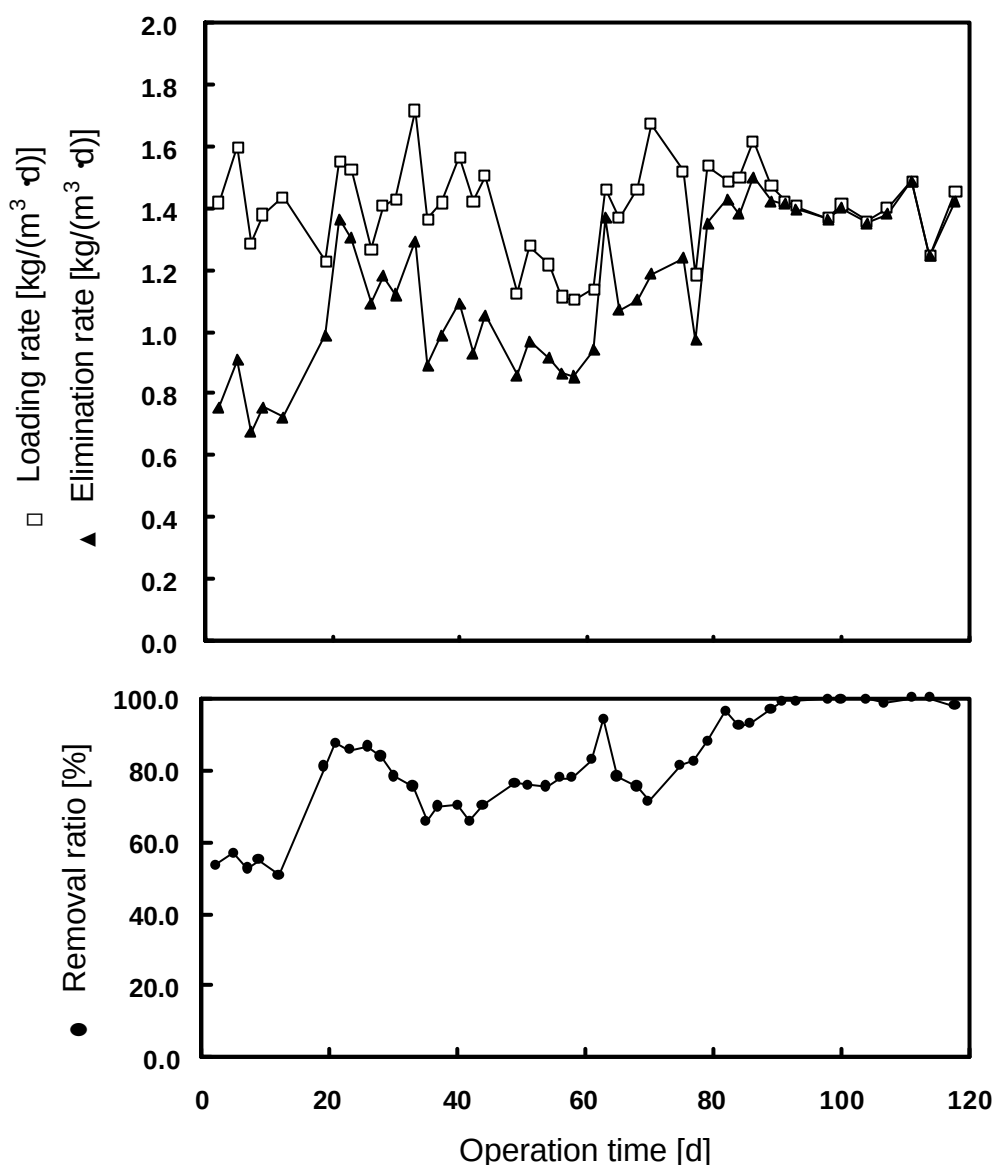


Fig. 2-8 Performance of the TBAB used for isolation of toluene-degrading bacteria about toluene elimination. Removal ratio was calculated from the ratio of toluene concentration in the effluent to that in the influent.

In order to examine the effectiveness of inoculation of *A. genospecies* Tol 5 for shortening of the adaptation period, initial performance of the TBAB inoculated with *A. genospecies* Tol 5 for toluene elimination was examined. As Fig. 2-9 shows, the removal ratio increased to reach near 80% within 24 h after starting the operation. The toluene elimination rate reached 1000 $\text{g}/(\text{m}^3 \cdot \text{d})$ soon. This suggests that by using *A. genospecies* Tol 5, a TBAB can be adapted in a short period to attain a desirable level of toluene elimination. This effectiveness is supposed to have been brought by not only toluene degradation ability but also adhesion ability of *A. genospecies* Tol 5. The strain had been immobilized onto the support by means of batch cultivation in presence of the support prior to the operation of the TBAB. Immobilized cells of the strain were not removed from the support by the sprinkle in the reactor during the operation. On the other hand, activated sludge could not be immobilized onto the support in a similar way. Therefore, activated sludge was resignedly added to the circulating medium. This way required the preliminary operation of the reactor for 7 d as mentioned in Experimental section. The timing of switching from the preliminary operation to the main operation for the toluene-elimination test was decided from the appearance of the support with adhered bacteria. Although the way of the preliminary operation such as its period or first inoculation volume should be considered so as to minimize the adaptation period in the case of use of activated sludge, the important point to note is that *A. genospecies* Tol 5 can be immobilized on support at the outside of a TBAB, which can work soon after packing the support with adhered cells without preliminary operation.

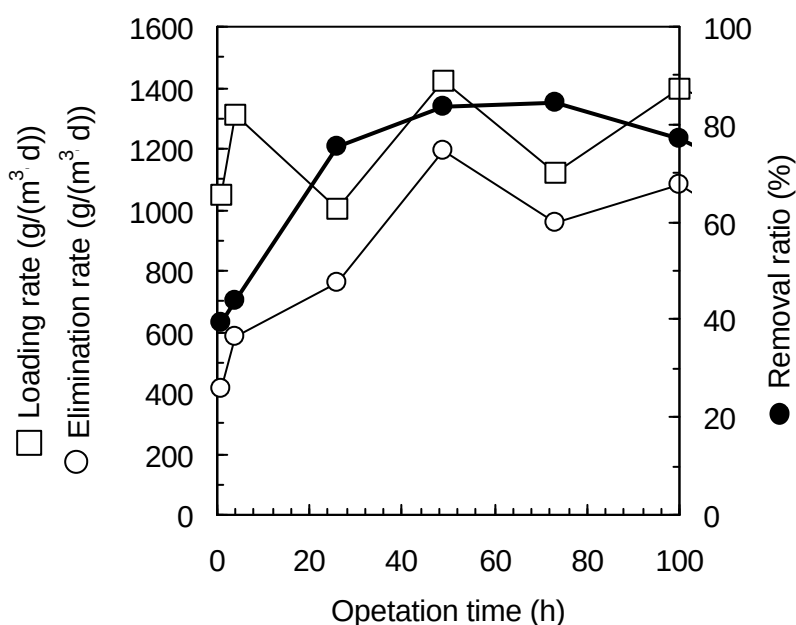


Fig. 2-9 Performance of the TBAB inoculated with *A. genospecies* Tol 5 about toluene elimination.

However, stability of toluene elimination ability in use of *A. genospecies* Tol 5 should be considered in a further study. As demonstrated in the performance of the TBAB inoculated with activated sludge, mixed culture is generally considered to show stable ability for a long period owing to its flexibility against change of environmental condition. Furthermore, during long-term operation of a TBAB in an open system, microbial species other than *A. genospecies* Tol5 might grow and the population of *A. genospecies* Tol 5 might decrease even though *A. genospecies* Tol 5 is used as a pure inoculum. In order to propose an effective way for use of *A. genospecies* Tol 5, the relationship between microbial flora including *A. genospecies* Tol 5 and toluene elimination ability during the long-term operation of the TBAB will be researched in the further study.

2-4. Summary

A. genospecies Tol 5 isolated from the TBAB which had been used for toluene elimination showed high toluene degradation ability, and had high adhesion ability to polyurethane. *A. genospecies* Tol 5 demonstrated the high efficiency in toluene removal. *A. genospecies* Tol 5 tended to adhere to hydrophobic materials showing water-repellent. When the bacterium was grown on toluene in presence of polyurethane foam, almost all cells adhered to the polymer support until the amount of 0.12 g-cell/g-polyurethane. Analysis of cell surface hydrophobicity by MATH tests and contact angle measurements revealed that *A. genospecies* Tol 5 has a noteworthy hydrophobic cell surface compared with *Escherichia coli*. A TBAB inoculated with *A. genospecies* Tol 5 showed a sufficiently high ability in toluene elimination within 24 h after starting the operation without preliminary operation for immobilization. This suggested that this strain was effective to shorten the adaptation period, which had been considerably long on the TBAB inoculated with original activated sludge.

Nomenclature

K_s	= saturation constant	[g/l]
s	= substrate concentration in a medium	[g/l]
t_d	= doubling time	[h]
v	= substrate removal rate	[mg-substrate/(g-cell min)]
$V_{\max}$	= maximum substrate removal rate	[mg-substrate/(g-cell min)]
$Y_{X/S}$	= growth yield	[g-cell/g-substrate]
θ_w	= contact angle against a water-droplet	[°]
$m_{\max}$	= maximum specific growth rate	[h ⁻¹]

Chapter III

Bacterial adhesion through cell surface appendages

3-1. Introduction

Microbial adhesion is detrimental to both human life and industrial processes, causing infection associated with medical materials [5, 14, 23, 82], dental decay [67], contamination of food [58], corrosion of metals [85], and so on. However, microbial adhesion can be also beneficial, for example, in the degradation of environmental hazardous chemicals [8, 29, 48] and in agricultural uses of root nodule bacteria [17]. Therefore, the mechanisms underlying microbial adhesion have attracted much attention from researchers in various fields [3, 7, 14, 67]. Tremendous amounts of works have been performed to investigate the natural process of bacterial adhesion to the various substrata over the years. Those works have shown two main factors for the bacterial adhesion processes. One of them is a physicochemical interaction between bacteria and material surfaces, such as Brownian motion, van der Waals attraction force, electrostatic interactions, and hydrophobic interaction. The other factor is a bridging function of bacterial surface polymeric structures, which include capsules, fimbriae (pili), flagella, and slime. These structures, so-called “adhesin” are responsible for firmer adhesion of bacteria to a surface [3, 7, 14].

Many researchers have shown that main factors responsible for tenacious bacterial adhesion are both exopolymeric substances (EPS), such as capsule and slime, and proteinaceous appendages, such as pili and flagella, through bridging between a cell and a surface (Table 3-1). Such cell appendages and EPS have been visualized by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Some researchers compared the difference of cell surface appendages between an adhesive wild type strain and its adhesion-deficient mutant using negative stained TEM image [16, 73]. Other reports showed the ultrathin section images of EPS bridging to the substratum at mature biofilm by using TEM [49, 23]. However, SEM is more adequate to observe the whole surface image of the interaction between bacterial cells and a substratum than TEM. Some researchers tried to observe the images of various bacterial adhesion by conventional SEM, for example, type IV pili of *Escherichia coli* [5], EPS of *Staphylococcus epidermidis* [18], and lateral flagella of *Vibrio parahaemolyticus* [4]. Recently, Kolari *et al.* showed that *Deinococcus geothermalis* adhered to the stainless steel through thin adhesion threads of EPS by field emission scanning electron microscopy (FE-SEM) for the first time [39]. These reports showed that many fibrils

Table 3-1 Bacterial cell appendage observed by the various instruments

Bacterial strain	Cell appendage	Instrument*		Reference
<i>Vibrio parahamolyticus</i>	Flagella	TEM [†]	Swarming	[86]
<i>Vibrio parahaemolyticus</i>	Flagella	SEM	Swarming	[4]
<i>Pseudomonas fluorescens</i>	Flagella	TEM [†]	Adhesin	[16]
<i>Pseudomonas putida</i>	Flagella	AFM	Biofilm	[25]
<i>Pseudomonas aeruginosa</i>	pili	TEM [†]	Receptor	[10]
<i>Pseudomonas syringae</i>	Hrp pili	TEM [†]	Secretion	[31]
<i>Escherichia coli</i>	type IV pili	SEM	Adhesin	[5]
<i>Pseudomonas aeruginosa</i>	type IV pili	TIRM	Adhesin	[78]
<i>Bacteroides gingivalis</i>	Fimbriae	TEM [†]	Adhesin	[26]
<i>Acinetobacter calcoaceticus</i>	Fimbriae	TEM [†]	Adhesin	[73]
<i>Saccharomyces cerevisiae</i>	Fibril	SEM	Network	[92]
<i>Deinococcus geothermalis</i>	Fibril	FE-SEM	Biofilm	[39]
<i>Pseudomonas</i> R3	Fibril	SEM	Adhesin	[50]
<i>Staphyrococcus epidermidis</i>	EPS	TEM [‡]	Biofilm	[23]
<i>Arthrobacter</i> strain	EPS	SEM	Biofilm	[60]
<i>Hyphomicrobium vulgare</i>	EPS	TEM [‡]	Adhesin	[49]
<i>Staphyrococcus epidermidis</i>	Slime	SEM	Biofilm	[18]
<i>Pseudomonas</i> strain	Glycocalyx	TEM [‡]	Biofilm	[13]
<i>Bacillus cereus</i>	N.O.*	VP-SEM	Biofilm	[57]

* Abbreviation

TEM: Transmission Electron Microscopy

SEM: Scanning Electron Microscopy

AFM: Atomic Force Microscopy

TIRM: Total Internal Reflection Microscopy

FE-SEM: Field Emission Scanning Electron Microscopy

VP-SEM: Variable Pressure Scanning Electron Microscopy

N.O.: not observed

[†] Observed in form of negative stain specimen[‡] Observed in form of ultra thin cut specimen

III. Cell surface appendages

of EPS or proteineous appendages randomly extending from the cell connect the cell body with the substratum.

It is difficult to reveal the real aspect of the interaction between a cell and a substratum with appendages by conventional SEM and TEM because of artifacts arising from sample preparation or contamination during observation. To overcome this difficulty, new technologies of SEM such as variable pressure scanning electron microscopy (VP-SEM, Hitachi) and environmental scanning electron microscopy (ESEM, Phillips) have been recently developed. VP-SEM and ESEM permit water-containing specimens to be observed in their natural or close to natural state, without requiring the conventional specimen preparations of fixation, dehydration, drying, and metal coating. Those instruments reduce water vaporization and beam damage by keeping the specimens at low temperature [2, 57]. However the resolution of these instruments are not enough to reveal the fibrous appendages of adhesive cells at scales in nanometer-scale.

In the previous chapter, it was shown that an adhesive bacterium, *A. genospecies* Tol 5, has a high ability to adhere to the polyurethane carrier. Presumably, *A. genospecies* Tol 5 is considered to possess the specific adhesin on its cell surface. The aim of this chapter is to reveal cell surface structure involved in bacterial adhesion by SEM, TEM, and novel developed microscopic technologies.

3-2. Materials and methods

3-2-1. Bacterial strain and culture

A. genospecies Tol 5 is used for a primary strain of this study (See Chapter II). *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 (Kyushu University, Japan). This plasmid harbors a gentamicin-resistant gene fragment, an RP4-specific *mob* site, and a transposon Tn5 derivative, Tn5-B30, containing a tetracycline-resistant gene fragment [77]. *Acinetobacter* strains were grown in 20 ml of BS medium (Table 2-1) supplemented with 10 μ l of toluene (equilibrated aqueous toluene concentration was 164 μ g/ml) in a 100-ml Erlenmeyer flask. To the flask, four pieces ($1.15 \times 1.15 \times 1.00$ cm) of sponge carrier made of polyurethane, with a 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 and 28°C. *E. coli* S17-1 was grown in Luria-Bertani (LB) medium (Polypeptone, 10 g; Yeast extract, 5 g; NaCl, 10 g; per liter; pH, 7.0) supplemented with tetracycline (12 μ g/ml) and gentamicin (15 μ g/ml) at 37°C. *Pseudomonas aeruginosa* PAO1, *Escherichia coli* O157: H7 (ATCC43888), and *Escherichia coli* JM109 were grown in 20 ml of LB medium in the presence of polyurethane carrier at 37°C.

3-2-2. Adhesion test

Cells were cultivated until stationary phase in the presence of polyurethane. The cells were harvested and resuspended in BS medium to provide an optical density at 660 nm (OD_{660}) of 0.5. Four pieces (6,400 mm^2 total surface area) of fresh polyurethane carrier were added to 20 ml of the cell suspension in a 100-ml Erlenmeyer flask. The cells and polyurethane carrier were incubated at 28°C with shaking at 115 rpm for 30 min in the absence of a carbon source so that the bacteria could not grow during incubation. After incubation for 30 min, the change in the OD_{660} value of the aqueous phase was analyzed. The adhesion ratio was calculated as follows:

$$\text{Adhesion ratio [\%]} = \frac{\text{OD}_{660\text{-initial}} - \text{OD}_{660\text{-after adhesion}}}{\text{OD}_{660\text{-initial}}} \times 100$$

The cell concentration corresponding to an OD_{660} of 0.5 and the total surface area of the carrier were 4.2×10^8 cells/ml and 6.4×10^3 mm^2 , respectively. Because the volume of the cell suspension was 20 ml, the specific number of adhering cells obtained from 100% of the adhesion ratio was 1.3×10^6 cells/ mm^2 .

3-2-3. Microscopy for observation of cell surface appendages

Field emission - scanning electron microscopy (FE-SEM)

All samples were obtained from cultures that had been grown to stationary phase in the presence of the polyurethane carriers, with toluene as the sole carbon source. The cells adhering to the polyurethane carriers were fixed with 1.25% glutaraldehyde in 0.067 M phosphate-buffered saline (PBS) (pH 6.5) for 1 h at 4°C, and then with 1.3% osmium tetroxide in 0.067 M PBS (pH 6.5) for 2 h at room temperature. After being dehydrated by a graded series of ethanol solution, the samples were subjected to critical point drying (HCP-2, Hitachi, Japan). The resultant specimens for SEM were coated with platinum and palladium using an ion sputter apparatus (E-1030, Hitachi, Japan). Cells suspended in liquid phase in the case of T1 cells and WT cells dispersed by trypsin treatment were harvested by slow centrifugation ($70 \times g$, 10 min). The harvested cells were fixed and subjected to the subsequent procedure for sample preparation as mentioned above. The specimens were observed under a S4700 FE-SEM (Hitachi, Japan) operating at 5-10 kV.

Variable pressure FE-SEM

All samples were obtained from cultures that had been grown to stationary phase in the presence of a carbon specimen stub or polyurethane carrier, with toluene as the sole carbon source. The cells that adhered to the substrata were fixed with 1.25% glutaraldehyde in 0.067 M PBS (pH 6.5) for 1 h at 4°C. Without any other pre-treatments, the specimens on the substrata were observed under a variable pressure FE-SEM (VP-FESEM) (Model S4300 SE/N; Hitachi, Japan) equipped with a cooling stage unit and an environmental secondary electron detector (ESED). The specimens were mounted in the specimen cooling stage, which was kept at -20°C . An accelerating voltage of 10 kV was used for VP-FESEM operating in a chamber with a pressure of 80 Pa.

Transmission electron microscopy (TEM)

All samples were obtained from cultures that had been grown to stationary phase in the presence of a polyurethane carrier, with toluene as the sole carbon source. The adhered cells were separated by vigorous shaking, and washed with fresh BS medium. Several drops of cell suspension were settled onto a collodion-coated copper grid of mesh size 200 (Nisshin EM, Japan) for 5 min. After brief wash with deionized water, the grid was immersed in 30 μl of filtered 2% phosphotungstic acid (pH 7.0) for 3 min. The grid was removed, and the excess stain was wicked away with filter paper. Then the grid was washed with deionized water and dried at the room temperature for 45 min. The specimens were observed under a H7500 TEM (Hitachi, Japan) operating at 80 kV without any other pre-treatments.

Atomic force microscopy (AFM)

Glass cover slips were cleaned in $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (70:30 volume%) for 1 h at room temperature and then rinsed with deionized water. Clean glass cover slips were made hydrophobic through immersion in 10 mM octadecyltriethoxysilane dissolved in toluene for 2 h followed by extensive rinsing with toluene. The glasses were heated to 80°C for 16 h. Water contact angles on the hydrophobic glasses were confirmed to be $>85^\circ$. The hydrophobic glass was immersed in the bacterial cell suspension for 1 min. The glass was removed, and dried by blowing with N_2 gas.

An AFM (SPN32, Hitachi, Japan) was operated in air in constant-amplitude mode, meaning that the oscillating tip approached the surface until a given amplitude reduction was reached during each period of the oscillation. The topography images in this study resulted from the cantilever movement in the z direction. The phase images show the phase shift that occurred between the free oscillation of the tip and the oscillation during surface touching, with the latter depending on the viscoelastic properties of the sample surface and on the adhesive potential between the sample and the tip.

3-2-4. Enzyme treatments and periodate oxidization

Tol 5 cells were cultivated to stationary phase in the presence of the polyurethane carrier. The carriers with adhered cells were collected, briefly washed, and treated with trypsin. The reactions were started by the addition of the enzyme to a final concentration of 0.25%. After incubation at room temperature for several minutes with shaking at 115 rpm, the samples were mixed vigorously by a vortex for 5 s.

The periodate oxidation was started by the addition of sodium periodate to a final concentration of 0.02 M [49]. After incubation at room temperature for 4 h with shaking (115 rpm), the turbidity of the solution was measured.

3-2-5. Surface protein analysis

Biotinylation and SDS-PAGE

Cell surface proteins were analyzed by an ECL protein biotinylation system (Amersham Pharmacia Biotech). Bacterial cells were collected by centrifugation and suspended in 1 ml of 0.067 M PBS (pH 7.5) containing 40 μl of biotinylation reagent. After incubation for 30 min at 4°C, the biotin-labeled cells were centrifuged at $1,900 \times g$ for 10 min and washed twice with PBS. The pellet was suspended in 0.2 ml of lysis buffer (250 mM NaCl; 25 mM Tris-HCl (pH 7.5); 5 mM EDTA; 2 $\mu\text{g}/\text{ml}$ aprotinin; 200 $\mu\text{g}/\text{ml}$ PMSF; 2% (v/v) SDS), incubated for 20 min at room temperature to complete cell lysis, and centrifuged at $13,000 \times g$ for 10 min to remove debris. To 50 μl of supernatant containing the

biotin-labeled surface proteins and eluted intracellular proteins, an equal volume of 2 × SDS gel-loading buffer (50 mM Tris-HCl (pH 6.5); 10% (v/v) glycerol; 2% (v/v) SDS; 2% 2-mercaptoethanol; 0.1% bromophenol blue) was added and boiled for 5 min. The resultant sample was run on an SDS-polyacrylamide (12%) gel and then electroblotted onto a PVDF membrane (Millipore). After blocking the membrane with Block Ace (Dainippon Pharmaceutical, Japan) for 1 h, streptavidin-conjugated horseradish peroxidase was bound to the biotin-labeled proteins by a 1-h incubation in Tween-PBS solution (0.14 M NaCl; 2.7 mM KCl; 8.1 mM Na₂HPO₄; 1.5 mM KH₂PO₄; 1% (v/v) Tween 20; 10% (v/v) block ace). The ECL Western blotting detection reagent was used for light emitting detection.

Two-dimensional gel electrophoresis

Total proteins were analyzed by a two-dimensional gel electrophoresis. Bacterial cells (10 mg) were collected by centrifugation, and suspended in 150 µl of lysis buffer (60 mM Tris; 5 M Urea; 1 M thiourea; 2 µg/ml aprotinin; 1% (w/v) CHAPS; 1% (v/v) Triton X-100; 1 particle of CompleteTM mini DNA free; 1% (w/v) dithiothreitol). The suspension was sonicated for 1 min at 0°C (20 kHz, 100 W) to complete cell lysis, and centrifuged at 100,000 ×g for 30 min at 4°C to remove debris. Thereafter 15 µl of 1 M acrylamide was mixed to the solution. The protein concentration was determined by Bradford method [9].

Two-dimensional gel electrophoresis was performed according to the manufacturer's instructions using a 2D compact System from (AE-6541, Atto, Japan). The agarGEL (Atto, Japan) was used for isoelectric focusing of the first electrophoresis. The e-PAGEL with a concentration gradient from 5 to 20% (E-D520L, Atto, Japan) was used for subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The total protein sample (100 µg) was overlaid to the agarGEL. Two-dimensional gels were stained with Coomassie brilliant blue R250.

3-2-6. Swarming and swimming tests

Swarming agar plates were prepared using M8 salt supplemented with 0.2% glucose and 0.05% glutamate, and solidified with 0.6% agar [37] (Table 3-2). Cells from single colonies were inoculated onto the middle of the swarming plates by a toothpick. *P. aeruginosa* PAO1 and *E. coli* O157: H7 (ATCC43888) were incubated at 37°C for 2 d. *A. genospecies* Tol 5 was incubated at 28°C for 2 d in a desiccator containing gas-phase toluene at a concentration of 0.2 mM.

Swimming plates were made of LB medium containing 0.25% agar and inoculated as above. *P. aeruginosa* PAO1 and *E. coli* O157: H7 were incubated at 37°C for 16 h. *A. genospecies* Tol 5 was incubated at 28°C for 16 h.

Table 3-2 Composition of M8 (GA) salt

Component	concentration [g/l]
Na ₂ HPO ₄	6.0
KH ₂ PO ₄	3.0
NaCl	0.5
ME solution	400 µl
MgSO ₄	2.0 mM

3-2-7. Transposon mutagenesis and selection of less-adhesive mutants

To generate transposon insertion mutants of *A. genospecies* Tol 5, the wild-type strain (WT) of Tol 5 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 cell (Tol 5), and subsequently Tn5-B30 was inserted into the chromosome of the recipient cell. Transformants can be readily selected on a plate containing tetracycline for the marker gene on Tn5-B30; however, it is difficult to select less-adhesive mutants from random transformants on a plate.

We therefore established a new method for the efficient selection of less-adhesive mutants. After conjugation, the filter with grown colonies was washed in 1 ml of 0.85% NaCl solution to recover all cells from the filter. 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 was grown under the same conditions except for the absence of tetracycline, the concentration of planktonic cells of WT 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 $\times 10^{-4}$ 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*; accession no. X75761) by PCR to confirm that they had been generated by the integration of Tn5-B30 into their chromosome.

3-2-8. Transposon mutagenesis and selection of less-adhesive mutants

Digoxigenin (DIG)-labeled probes were prepared by using a PCR DIG-Probe-Synthesis kit (Roche Diagnostic) according to the manufacturer's instructions. The probe DIG-*tet A*, which was specific for *tet A* of Tn5-B30, was generated with primers (5'-GTGAAACCCAACATAACCCCTGAT-3' and 5'-GCGATCGGCTCGTTGCCCTG-3') and the template pSUP102Gm::Tn5-B30, which was extracted from *E. coli* S17-1. Chromosomal DNAs extracted from *A. genospecies* Tol 5 and its mutants were digested with *Eco*RI or *Hind*III and separated by electrophoresis on a 0.65% agarose gel. The agarose gel containing DNA was soaked in denaturation solution (0.5 M NaOH; 1.5 M NaCl) for 15 min and then washed in neutralization solution (1.0 M Tris; 1.5 M NaCl, pH 8.0) for 15 min. DNA was transferred onto a nylon membrane (Millipore) by capillary blotting with 20×SSC (3 M NaCl; 0.3 M sodium citrate, pH 7.0) and was fixed to the membrane by UV cross-linking at 1,200 J/cm². For prehybridization, the membrane was treated with DIG-Easy-Hyb (Roche Diagnostic) and incubated at 55°C for 30 min. For hybridization, it was incubated with the DIG-labeled probe DNA at 55°C overnight. The membrane was washed twice in 2×SSC plus 0.1% (w/v) sodium dodecyl sulfate (SDS) for 5 min each at room temperature, and twice in 0.1×SSC plus 0.1% (w/v) SDS for 15 min each at 68°C. Thereafter, the hybridized probes were detected by using a DIG-Luminescent-Detection kit (Roche Diagnostic) according to the manufacturer's instructions.

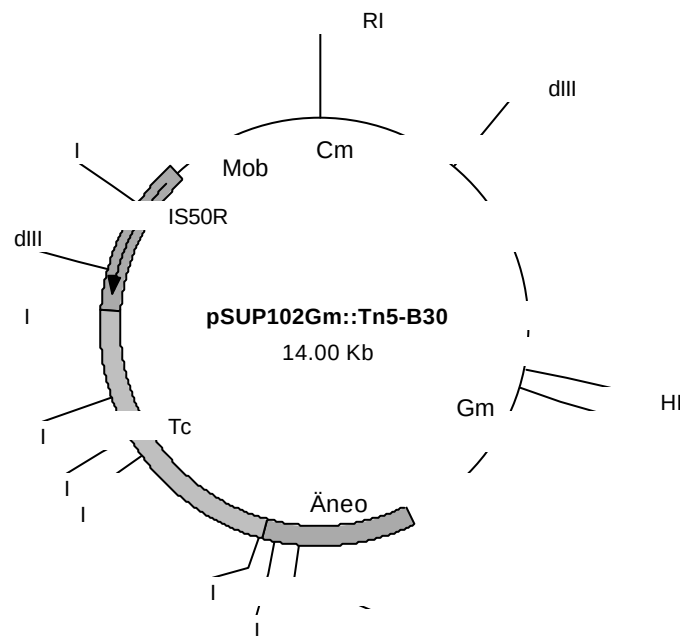


Fig. 3-1. pSUP102Gm::Tn5-B30 is a pACYC184 derivative with RP4-specific *Mob*-site and transposon Tn5-B30 (*Tc*^R). Arrow in pSUP102Gm::Tn5-B30 is transposase gene.

3-3. Results and discussion

3-3-1. Adhesion property of *A. genospecies* Tol 5

The strong adhesive property of *A. genospecies* Tol 5 was examined by the adhesion test. During the 1 h incubation, more than 99% of the cells adhered to the polyurethane carrier. The number of adhered cells was nearly 1.3×10^6 cells/mm² (Fig. 3-2). On the contrary, when *P. aeruginosa* PAO1, a representative strain of adhesive bacteria, was subjected to the same adhesion test, only 12.6% of the cells (1.6×10^5 cells/mm²) adhered to the polyurethane. *E. coli* JM109 was hardly adhered at all. The adhesiveness of *A. genospecies* Tol 5 was 10-fold higher than that of *P. aeruginosa* PAO1.

In numerous studies on adhesive bacteria, adhesion to a substratum has been reported to be in the range of 10^4 - 10^5 cells/mm² [28, 45, 49, 66]. For example, the adhesiveness of *P. aeruginosa* PAO1 to a glass surface, which has been studied as a representative strain of adhesive bacteria, was shown to be 1.2×10^5 cells/mm² [66]. Despite differences in the substratum and experimental conditions, the datum on *P. aeruginosa* PAO1 adhesiveness (1.6×10^5 cells/mm²) corresponds closely to this value. This result implies the validity of our method for comparing the adhesiveness of various bacterial strains.

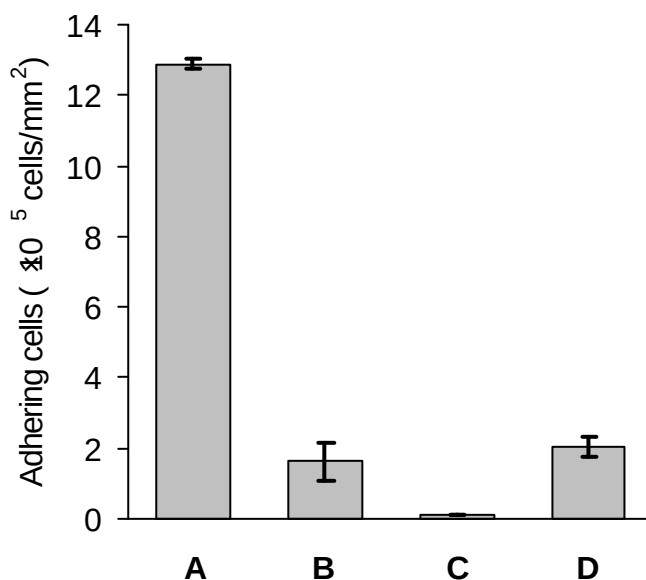


Fig. 3-2 Specific numbers of adhering cells during adhesion tests.

A: *Acinetobacter genospecies* Tol 5. **B:** *Pseudomonas aeruginosa* PAO1.

C: *Escherichia coli* JM109. **D:** T1, a less-adhesive mutant of *A. genospecies* Tol 5

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After growing the Tol 5 cells in a liquid medium in the presence of polyurethane carrier, the surface of the polyurethane carrier with adhered cells was observed by field emission scanning electron microscopy (FE-SEM). The micrographs revealed that, during growth in the presence of polyurethane, Tol 5 cells adhered to the substratum of polyurethane in the form of multi-layer cell clusters (Fig. 3-3, A). By the vigorous shaking, Tol 5 cells adhered to the carrier were partly separated from the carrier in the form of large cell aggregates (Fig. 3-3, B). The micrographs show that the strong adhesive property of Tol 5 is caused by 'co-adhesion' [7], that is, the subsequent adhesion of cells to the cells that initially adhered to a substratum.

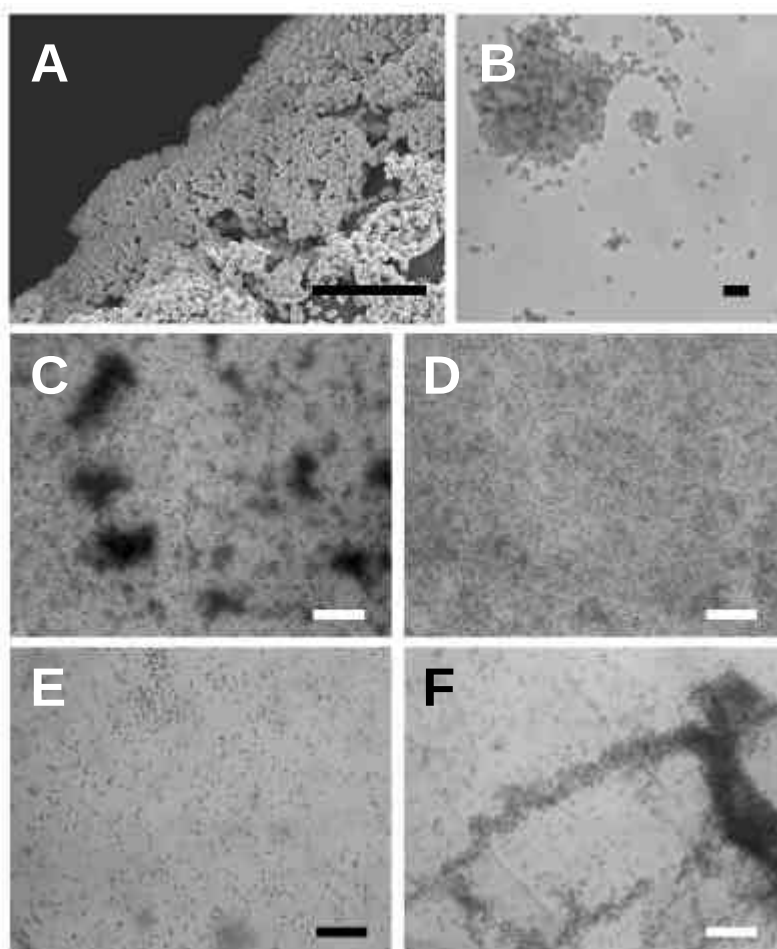


Fig. 3-3 (A) Adhesion to the polyurethane carrier of Tol 5 cells at the stationary phase under FE-SEM. (B) Large cell aggregates separated from the carrier by vigorous shaking. Tol 5 cells were cultivated to the stationary phase in the presence of the glass cover slip (C) or mica (D). *P. aeruginosa* PAO1 cells were cultivated to the stationary phase in the presence of the glass cover slip (E) or mica (F) in LB medium. Bars = 10 μm.

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In addition, Tol 5 cells adhered to a variety of abiotic substrata, such like mica, glass, polypropylene, PTFE film, and epoxy resin (data not shown). This indicates that adhesion of Tol 5 cells to solid surface is non-specific. After growing the Tol 5 cells in a liquid medium in the presence of glass cover slip or mica, the surface of the substratum with adhered cells was observed by light microscopy (Fig. 3-3 C-D). To these hydrophilic substrata, Tol 5 cells also adhered to the substratum in the form of large aggregates, thereby determination of cell adhesion by direct counting on substrata is difficult for Tol 5 cells. On the contrary, *P. aeruginosa* PAO1 cells adhered to the glass cover slip or mica apparently less than Tol 5 cells (Fig. 3-3 E-F).

3-3-2 Identification of cell-surface appendages of Tol 5 cells

We observed the cell surface structure of Tol 5 cells at high resolution using transmission electron microscopy (TEM). Compared with SEM, TEM provides high-resolution images of cell surface appendages by operating at higher electron-accelerating voltages (usually 70-100 kV for biotic samples). In particular, in a negative staining method for TEM observation, the sample is mounted on a collodion-coated copper grid and subjected to electron staining with phosphotungstic acid, uranyl acetate, and/or ruthenium red, without any other pretreatment. Therefore, this microscopic method gives images reflecting intact morphology of cell surface structures unaffected by sample preparation, although it is difficult to directly observe the real feature of the interaction between the cell and the substratum because of its projected image. Indeed, many researchers have shown bacterial cell surface appendages involved in cell adhesion by the combination of negative staining and TEM observation [16, 26, 73].

TEM images of Tol 5 cells negatively stained with phosphotungstic acid are shown on Fig. 3-4 (A-F). These images showed the existence of two major types of appendages, “anchor-like appendage” with thick, long, and strait features (Fig. 3-4 A-C), and “pili-like appendage” revealed peritrichate, thin, bent, and twisted features (Fig. 3-4 C-E). The anchor function as long-distance connectors between two cells, extending more than 300 nm in many cases (Fig. 3-4 A, B), but the pili-like appendages are protruded randomly from the cell (Fig. 3-4 C, D). Although some pili-like appendages connected with two cells (Fig. 3-4 E), the other pili-like appendages scarcely connected them (Fig. 3-4 C-D). Typically, a Tol 5 cell possesses a few numbers of anchors connecting with several other cells. It should be noted that only one anchor is involved in a pair of cell-cell connection. In contrast, peritrichate pili-like appendages were produced in large quantities per cell. Because of the projected image by negatively stained TEM, these appendages could not be revealed in the large aggregates of Tol 5 cells (Fig. 3-4 F).

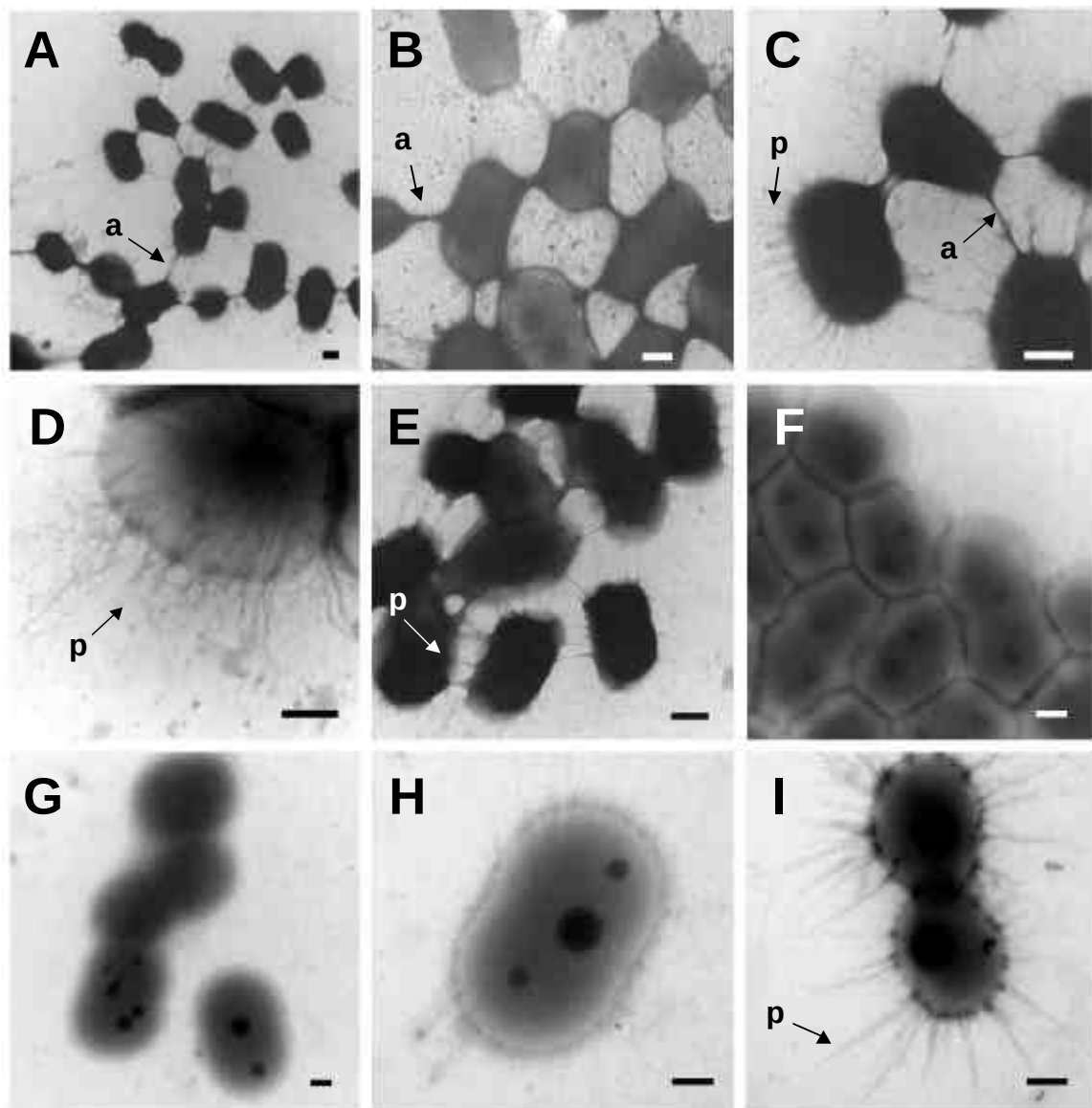


Fig. 3-4 Representative images of cell surface appendages of Tol 5 by TEM. The cells were mounted on a collodion-coated copper grid and negatively stained with phosphotungstic acid (**A - F**) or ruthenium red (**G - I**). (**A - C**) Tol 5 cells are connected each other with thick anchor-like appendage (**a**). (**C - E**) The thin pili-like appendages (**p**) were also shown on Tol 5 cells. (**D**) Their high production can be seen with their bent, twisted, and peritrichate morphology. (**F**) The large cell aggregate was shown in the Tol 5 cells. (**G, H**) These appendages were not stained by ruthenium red. (**I**) Pili-like appendages are not stained, but the appendages are revealed by residual ruthenium red. Bars = 300 nm.

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To clarify the difference between the anchor and pili-like appendage, the length and diameter of these two types of appendage were statistically analyzed on enlarged microphotographs by TEM. The anchor is 378 nm in average length, and 37 nm in average diameter ($n = 80$) (Fig. 3-5). The pili-like appendage is 286 nm in average length, and 10 nm in average diameter ($n = 111$) (Fig. 3-5). The statistical significance in differences between the anchor and pili-like appendage was in a significance level of $P < 0.05$ from a result of two-tailed Student's t - tests (Fig. 3-5).

By TEM, we also observed Tol 5 cells negatively stained with ruthenium red, which is used for staining saccharic components on the bacterial cell surface, such as lipopolysaccharide (LPS), capsule, and extracellular glycoproteins [41, 46, 80]. Both anchor and pili-like appendages were less emphasized by the negative staining with ruthenium red than with phosphotungstic acid (Fig. 3-4 E, F). Especially, unstained pili-like appendages were stroke up by residual ruthenium red (Fig. 3-4 I). This suggests that the anchor and pili-like appendage of Tol 5 cells do not mainly consist of saccharic compounds.

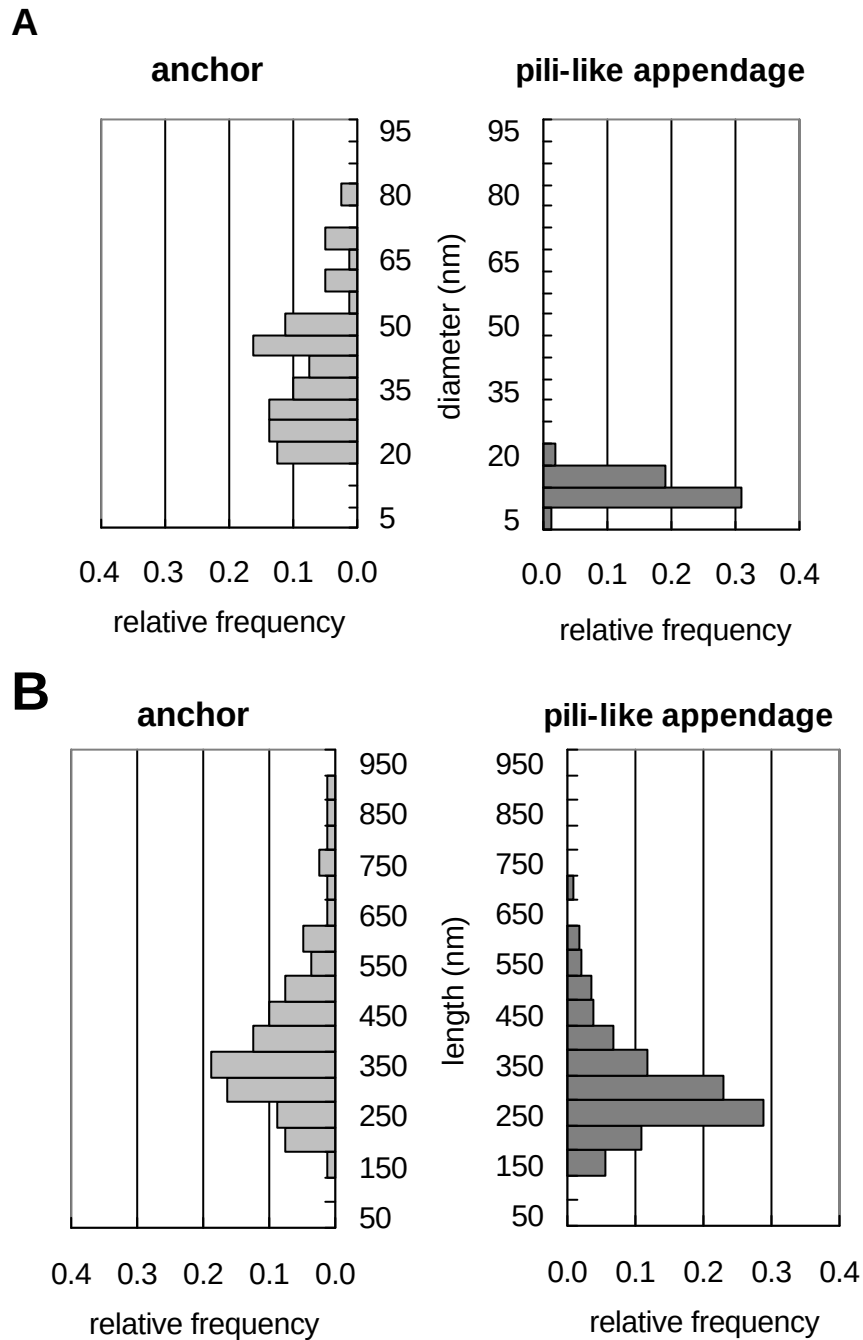


Fig. 3-5 Distribution of the diameter (**A**) and length (**B**) of the anchor and pili-like appendage. Eighty anchors and 111 pili-like appendages were statistically analyzed on enlarged images. The anchor is 378 nm in average length, and 37 nm in average diameter. The pili-like appendage is 286 nm in average length, and 10 nm in average diameter.

3-3-3. Steric morphology of the appendages for bacterial adhesion

While images obtained from TEM observation of negatively stained samples are planar projected images, those obtained from SEM are stereoscopic. Therefore, SEM is more adequate to observe the whole surface image of the interaction between bacterial cells and a substratum than TEM. Recently, Kolari *et al.* showed that *Deinococcus geothermalis* adhered to the stainless steel through thin adhesion threads of EPS using field-emission scanning electron microscopy (FE-SEM) for the first time [39]. The report showed that cell bodies and a substratum are connected with many fibrils of EPS or pili randomly extending from the cell. In comparison with conventional SEM, FE-SEM provides improved spatial resolution by the use of a field-emission electron source, minimizes sample charging up and damage by operating at lower electron-accelerating voltages, and gives better images of immediate biological surfaces owing to a reduction in electron penetration [61].

The adherence of Tol 5 cells to the polyurethane surface was observed at high resolution by FE-SEM. FE-SEM of the cell surface also revealed the existence of anchor-like appendages on Tol 5 cells (Fig. 3-6 A-C). The images clearly show that the end of this anchor directly interacts with the substratum, tethering the body of the cell to the surface (Fig. 3-6 C). The anchors fasten the cells like a string with a balloon attached at the opposite end (Fig. 3-6 B). They also function as long-distance connectors between two cells, extending more than 500 nm in many cases (Fig. 3-6 A). The peritrichate pili-like appendages were also identified on Tol 5 cells by FE-SEM (Fig. 3-6 D-H). Their bent, twisted, and bundled like features are a little different from their images obtained by TEM, because their morphology might be affected by sample preparation including dehydration and by thickening through heavy metal coating during ion sputtering. The pili-like appendages by SEM seem thicker than by TEM probably due to contamination, which is an inevitable drawback in SEM (Fig. 3-4 C-E, Fig. 3-6 E, H). Although the pili-like appendages interact directly with the substratum, the interaction does not occur at the end of the appendages as it does in the anchor. Instead, the interaction between pili-like appendages and substratum extends along a large part of the appendage (Fig. 3-6 F, G). Furthermore, in order to attain tight cell clusters, cells that form large aggregates appear to produce large numbers of pili-like appendage (Fig. 6 D).

While the long distant interactions between a cell and a substratum or another cell mediated by the anchor form a flexible cell network on the substratum (Fig. 3-6 A, B), the pili-like appendage seems to cause dense cell aggregations to develop a tenacious biofilm (Fig. 3-6 D). Thus, FE-SEM images suggest that both anchor and pili-like appendages are probable to play important roles for strong adhesion of Tol 5 cells.

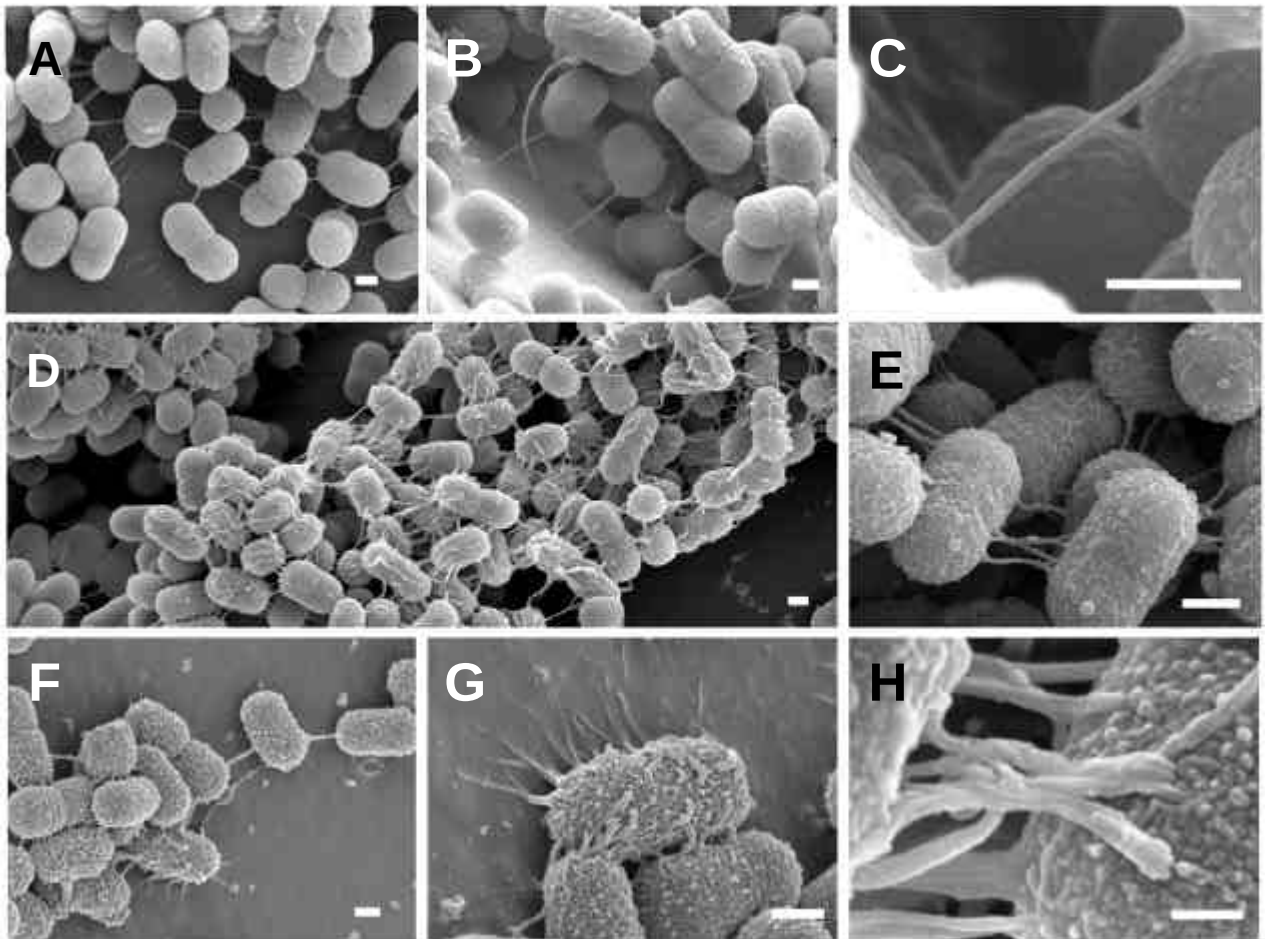


Fig. 3-6 Representative images of cell surface morphology of Tol 5 by using FE-SEM. Tol 5 cells are connected each other (A) and tethered to the substratum (B) with anchor-like appendages. The close-up image of the vicinity of anchored region shows the direct interaction of the anchor with the substratum at its end (C). The pili-like appendages are also shown on the surface of Tol 5 cells (D to H). Their high production can be seen on the large cell aggregation (D). The close-up images show their bent, twisted, or branched morphology (E, H). Tol 5 cells tightly adhered to the substratum with peritrichous pili-like appendages (F, G). Bars = 300 nm except panel H (bar = 100 nm).

3-3-4. Validation of steric morphology by VP-FESEM

The unique morphology of the anchor was confirmed using a VP-FESEM/ESED system. This instrument permits water-containing specimens to be observed for several hours in their natural or close to natural state, without requiring the conventional specimen preparations of osmium fixation, dehydration, drying, and metal coating. It reduces water vaporization and beam damage by keeping the specimens at low temperature [57]. In addition, in spite of the presence of gas molecules, ESED allows microscopy using secondary electrons, which enabled us to observe the surface structure of intact cells at higher resolution than can be achieved by a conventional VP-SEM equipped with a back-scattered electron detector. The secondary electrons generated from the sample travel in the specimen chamber and collide with residual gas molecules, generating cation and electron pairs. The electrons are accelerated toward the electrode, ionizing more gas molecules as they travel, whereas the cations move toward the sample surface, which is negatively charged by an electron beam, are collected on it and are detected in the form of an ion current flowing through the sample.

Callow *et al.* tried to reveal the spore adhesive of the marine alga in its natural hydrate state using field-emission environmental scanning electron microscopy (FEG-ESEM, Phillips) [11]. They showed a swollen gel-like adhesive pad in contrast to the fibrous adhesive imaged by conventional SEM [11]. This instrument has almost the same performance as VP-FESEM, but there is no report that has shown images of fibrous appendages on the bacterial surface by this instrument. Hence, this is the first observation to show nearly ‘real’ (that is, natural state) images of bacterial cell surfaces and appendages obtained by VP-FESEM/ESED.

The observation of Tol 5 cells by VP-FESEM proved that the intact anchor does indeed have a long, straight morphology (Fig. 3-7 A-C, E). The cell-cell connection through anchor was also revealed by VP-FESEM observation (Fig. 3-7 A, C). Correspondingly, the bridging function of the anchor between the cells and the substratum was validated (Fig. 3-7 B, E). Moreover, the pili-like appendages of Tol 5 cells were also identified by VP-FESEM, although the resolution is not so high compared with usual FE-SEM (Fig. 3-7 D). These results by using VP-FESEM revealed the steric morphologies through appendages as same as the images by using FE-SEM. Hence these specific stereoimages of anchor and pili-like appendages are not artifacts of sample preparation.

In addition to the long straight morphology, only the very end of the anchor interacts with the substratum, which enables the cell to adhere at a ‘long’ distance, that is, several hundreds of nanometers from the substratum. In order to elucidate mechanisms underlying bacterial adhesion, the distance between a cell and a substratum is important knowledge. Although this distance has been estimated by the calculation based on the physicochemical model, results of direct measurement of the distance has been lacking [7, 94]. Recently,

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Vigeant *et al.* reported the *Escherichia coli* adhered to the substratum through “tethered” state by its flagellum. They tried to measure the absolute distances between the bacteria and the substratum by using a total internal reflection aqueous fluorescence (TIRAF) microscopy [94]. However, the curvature and the orientation of each individual cells made an uncertainty to the determination by TIRAF [79]. Scattering of laser light and roughness of the bacterial surfaces also contribute to the overestimation [79]. This is first report to reveal the tethered cell to the substratum through the tip of the anchor for bacterial adhesion by FE-SEM and VP-FESEM. It is considered that this aspect of bacterial adhesion is correlated with strong adhesiveness of the Tol 5 cell.

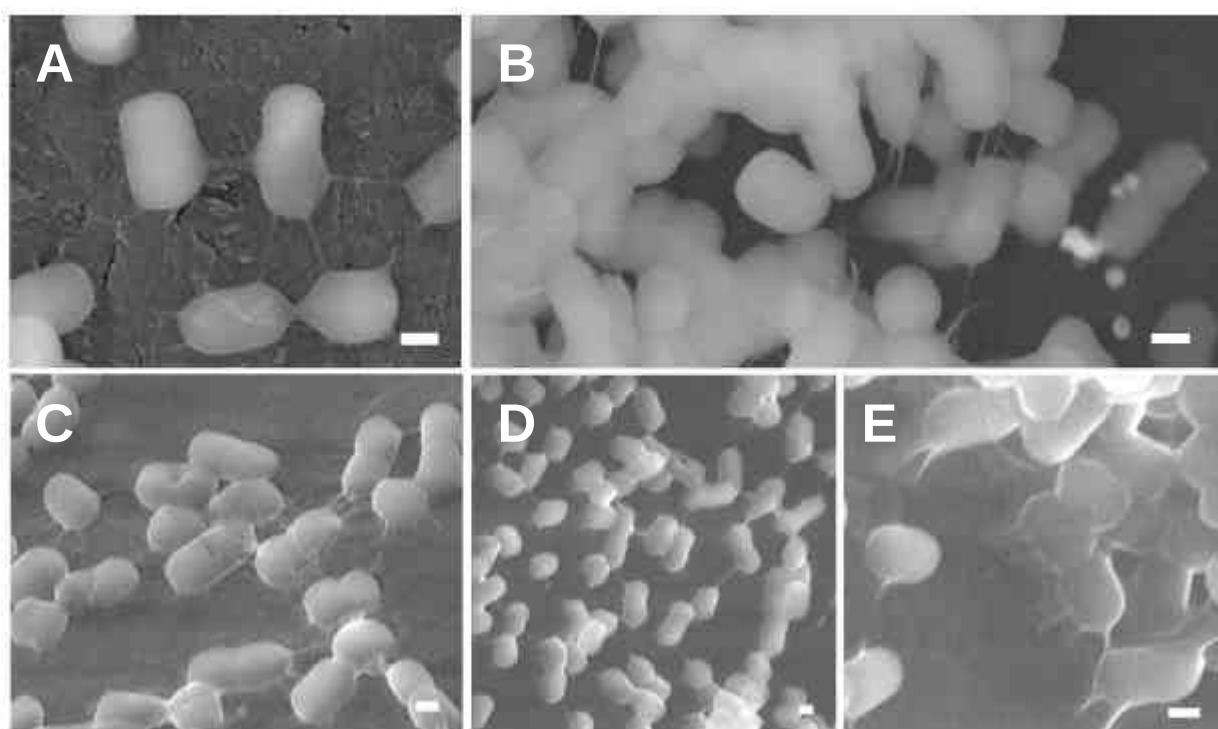


Fig. 3-7 Representative images of the cell-surface morphology of Tol 5 obtained using VP-FESEM. Cells were grown in the presence of a carbon specimen stub (**A, B**) or the polyurethane carrier (**C to E**) to allow them to adhere to the solid surface during growth. After growth, the carrier and stub with the adhered cells were collected. For observation by VP-FESEM, no pre-treatments other than glutaraldehyde fixation were carried out. Bars = 300 nm.

3-3-5. Characterization of the cell-surface appendages

To obtain information about chemical constituents of the appendages, Tol 5 cells were subjected to different polymer hydrolysis treatments: trypsin treatment, which is usually used to peel animal cells grown on anchorage from the substratum [88]; and periodate oxidation, which disrupts extracellular adhesive materials consisting of polysaccharides [49, 52]. Of these two treatments, treatment with trypsin for only 15 min dispersed the Tol 5 cells from the carrier into individual cells (Fig. 3-8 A-B). The vigorous vortex without trypsin hardly made the Tol 5 cells separated from the carrier. FE-SEM of the cells after trypsin treatment showed that trypsin had digested the appendages, producing debris (Fig. 3-8 C). The body of the cells did not suffer serious damage from trypsin because the cellular shape maintained a bacillar form. FE-SEM of the cells after only vortex treatment without trypsin did not show the destruction of appendages (data not shown).

Anchor and pili-like appendage were not stained by ruthenium red (Fig. 3-4 E, F) and the morphology of them looked proteinaceous in nature. The result of trypsin treatment and periodate oxidation also suggests that proteins but not polysaccharides are main components of the appendages, although it cannot be ruled out that polysaccharides constitute the appendages as minor components.

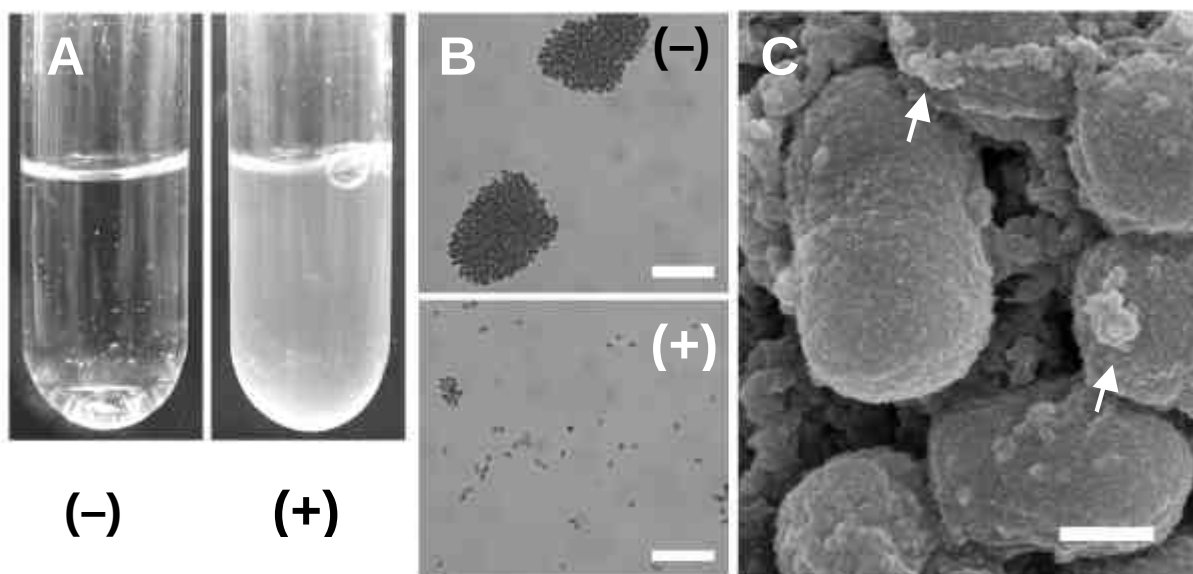


Fig. 3-8 Effect of trypsin treatment on adhered cells of Tol 5 to the carrier. (A) Culture solution of Tol 5 treated with trypsin (+) and a control without trypsin (-) after removal of polyurethane carriers. (B) Light microscopy images of the cell aggregation (-) and the cells dispersed by trypsin treatment (+). Bars = 25 μ m. (C) The surface of the cells dispersed by trypsin treatment observed by FE-SEM. White arrowheads indicate the likely debris from the appendages. Bar = 300 nm.

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We also examined the motility of *A. genospecies* Tol 5 because in many bacterial species motility arising from cell-surface appendages is important for cell adhesion [59, 65, 94]. Many bacteria are able to swim in plates comprising low concentrations of agar (<0.4%) by means of a polar flagellum [37]. In addition, several Gram-negative bacteria show swarming behavior on a soft agar plate (0.6-0.7%) [19, 37]. Using these specific types of agar plate, we therefore compared the swimming and swarming motilities of *A. genospecies* Tol 5 with those of both *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 flagella (H-antigen) for swimming. These motility tests clearly revealed that *A. genospecies* Tol 5 cells possess neither swimming nor swarming motility, whereas the expected motilities of *P. aeruginosa* PAO1 and *E. coli* O157: H7 were confirmed (Fig. 3-9). This suggests that the anchor on strain Tol 5 does not afford motilities such as swimming and swarming to the cells.

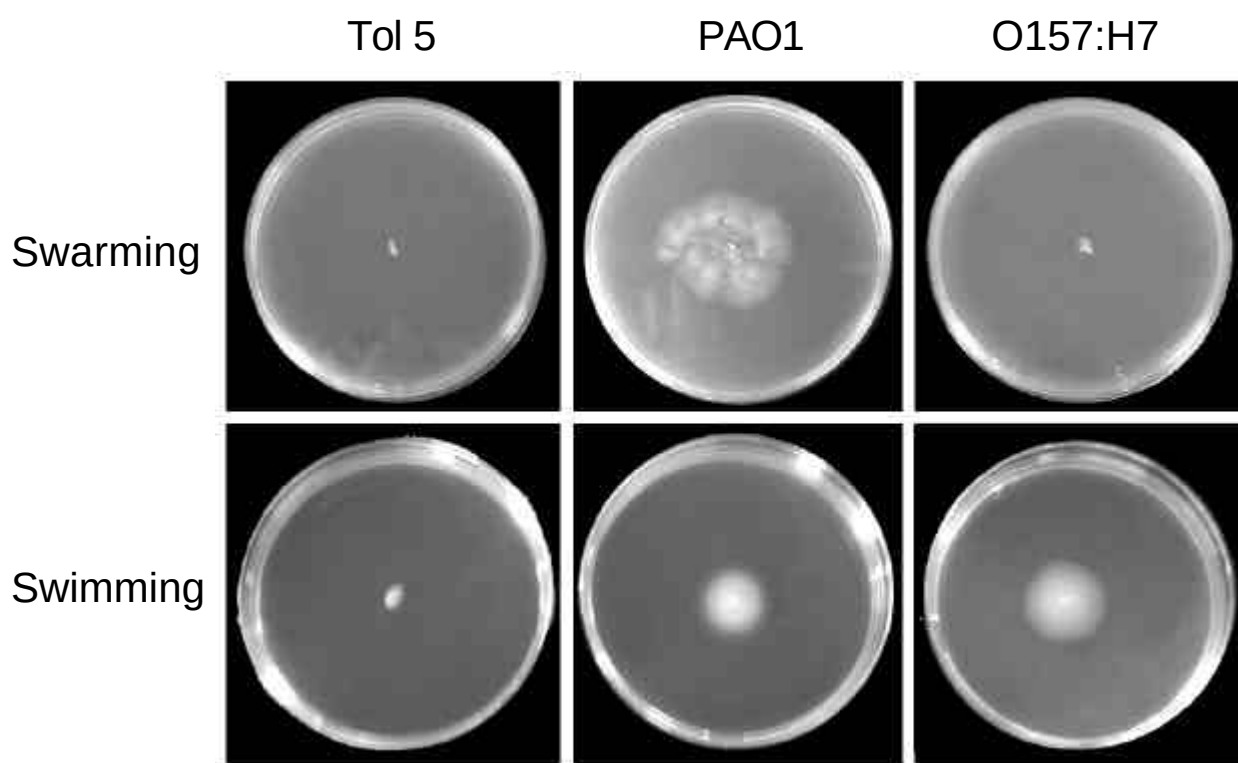


Fig. 3-9 Motility of the wild-type strains of *A. genospecies* Tol 5 (**Tol 5**), *P. aeruginosa* PAO1 (**PAO1**) and *E. coli* O157: H7 (**O157: H7**). Swarming ability was tested on M8/glucose/glutamate plates containing 0.6% agar. Swimming ability was tested on LB plates containing 0.25% agar. The plates were incubated for 2 days and for 16 h in the swarming tests and the swimming tests, respectively.

Although the anchor resembles type IV pili, which are required for adhesion to a substratum and for cell aggregation in many kinds of bacteria [5, 47], unlike type IV pili the anchor does not extend from the pole of a cell [95]. Moreover, although many previously reported cell-surface appendages have been shown to participate not only in adhesion but also in motility – such as swimming via polar flagella in many bacteria, and swarming via peritrichous flagella in *E. coli*, *Serratia marcescens* and *Pseudomonas mirabilis* [19] or via type IV pili in *P. aeruginosa* PAO1 [37] – *A. genospecies* Tol 5 shows no motility. Consequently, we propose that the anchor represents a unique, previously unreported cellular appendage.

3-3-6. Screening of less adhesive mutants of Tol 5

To prove that the unique appendages found on the cell surface of *A. genospecies* Tol 5, its less-adhesive mutants were obtained by transposon mutagenesis. The selection of less-adhesive mutants of Tol 5 is difficult because it is hard to identify such mutants through the appearance or morphology of their colonies on an agar plate, unlike bacterial strains that produce high levels of EPS. For bacteria such as Tol 5, for which it is difficult to select mutants on the basis of appearance, so far more than 1,000 transposon-inserted mutants have been subjected to routine screening of the individual liquid culture of each colony picked up to examine adhesiveness [27, 45]. In this study, an effective screening method was established to obtain less-adhesive mutants. The mutant library was accumulated in the bulk liquid of the medium during cultivation in the presence of the carrier, followed by suspended mutants were picked up. This method is much simpler than conventional screening schemes, and enables to be applied widely to the efficient screening for mutants that have lost adhesiveness to a solid surface.

Seven different mutants were obtained by seven times trial of transposon mutagenesis. The five mutants of them (T1 - T4, T6) showed dispersive cell feature and less adhesiveness, and the other two mutants retained the adhesive property (T5, A1) (Table 3-3). To determine the insertion site of Tn 5 in the chromosome of Tol 5, Southern blot analysis was carried out using the *tet A* gene as a probe. Unfortunately, however, the result showed that the insertion had occurred not at a single site but at multiple sites, including a homologous recombination site shared not only with less-adhesive mutants but with all mutants expressing tetracycline resistance (Fig. 3-10).

Table 3-3 Analysis of adhesion property and cell surface hydrophobicity of less-adhesive mutants

Bacterial strain	Adhesion ratio (%) [†]	Cell adhesion (×10 ⁵ cells/mm ²)
Wild-type	98	12.7
T1	15	1.9
T2	24	3.1
T3	12	1.6
T4	12	1.5
T5	96	12.5
T6	41	5.4
A1	97	12.6

[†] Adhesion ratio (%) = $(OD_{660\text{-initial}} - OD_{660\text{-after adhesion}}) / OD_{660\text{-initial}} \times 100$

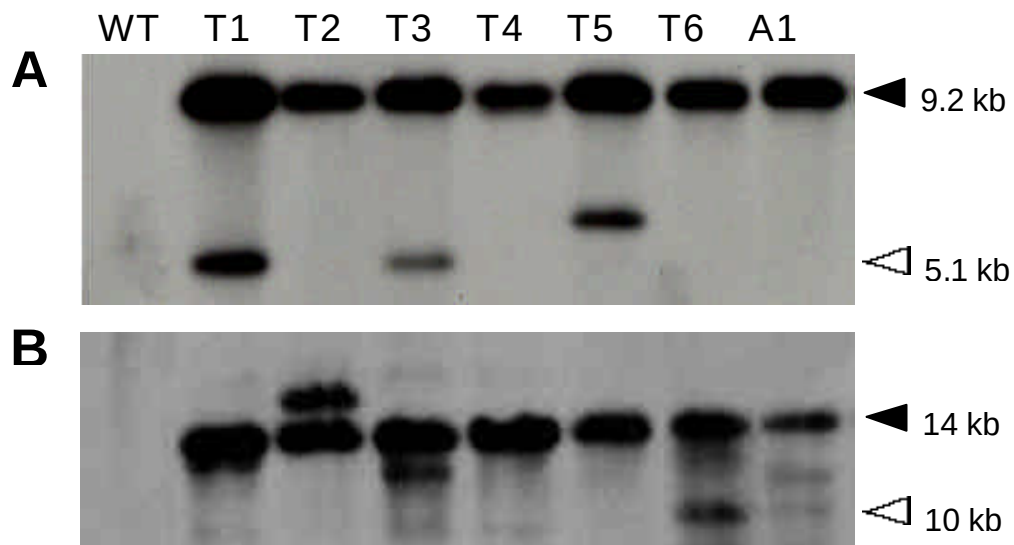


Fig. 3-10 Southern hybridization of less-adhesive mutants. Chromosomal DNA of each strain was digested with *Hind*III (A) or *Eco*RI (B). Fragments were separated on a 0.65% agarose gel. A luminescent detection was carried out after Southern transfer and hybridization with DIG-labeled *tetA*. Solid triangles represent likely homologous recombination bands. Open triangles represent one of bands inserted randomly.

3-3-7. Cell surface appendages of less-adhesive mutants

Among the less-adhesive mutants, T1, showed markedly reduced adhesiveness as well as T3 and T4, with only 15% of the cells (1.9×10^5 cells/mm²) adhered to the polyurethane (Table 3-3). When T1 was grown in liquid medium in the presence of polyurethane carrier, T1 was well-suspended in the liquid with the cells individually separated (Fig. 3-11 A, B). After growing T1 in the presence of polyurethane carrier, the surface of the polyurethane carrier with adhered cells was observed by FE-SEM (Fig. 3-11 B). The micrographs revealed that T1 hardly adhered to the polyurethane surface at all. Also, to glass cover slip or mica, T1 cells adhered less than wild-type (WT) (Fig. 3-11 C-D). These images clearly showed that adhesion ability of T1 cells were decreased by transposon mutagenesis. These results suggest that T1 cells have lost a specific adhesin to obtain strong adhesion ability by transposon mutagenesis.

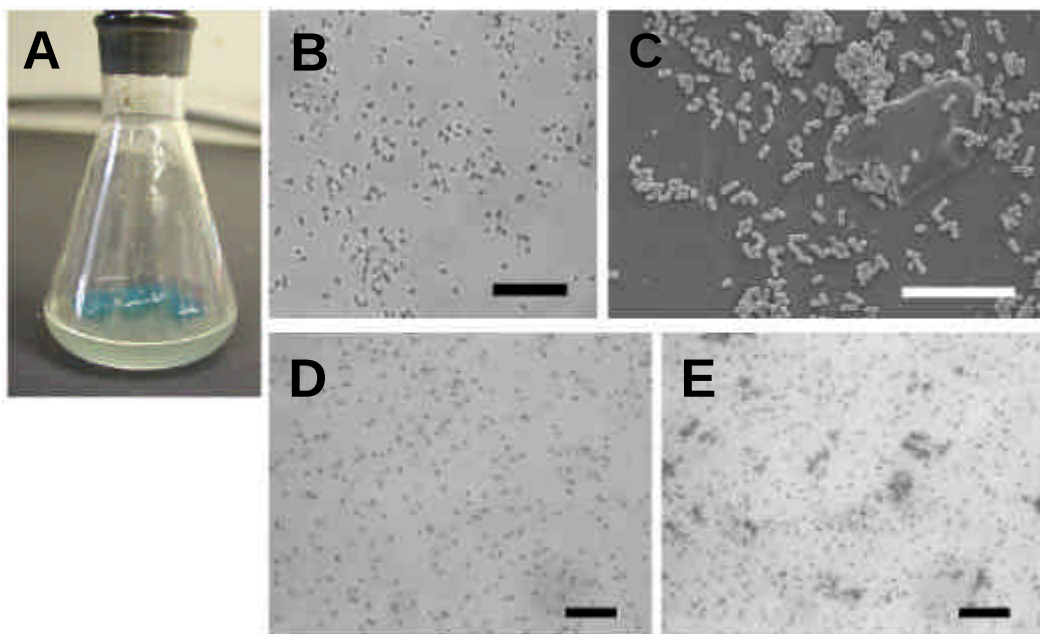


Fig. 3-11 (A) T1 cells were cultivated to the stationary phase at the BS medium supplied with toluene. (B) Cell suspension in the liquid phase of less-adhesive T1 mutant. (C) Adhesion to the polyurethane carrier of T1 cells at the stationary phase under FE-SEM. T1 cells were cultivated to the stationary phase in the presence of the glass cover slip (D) or mica (E). Bars = 10 μ m.

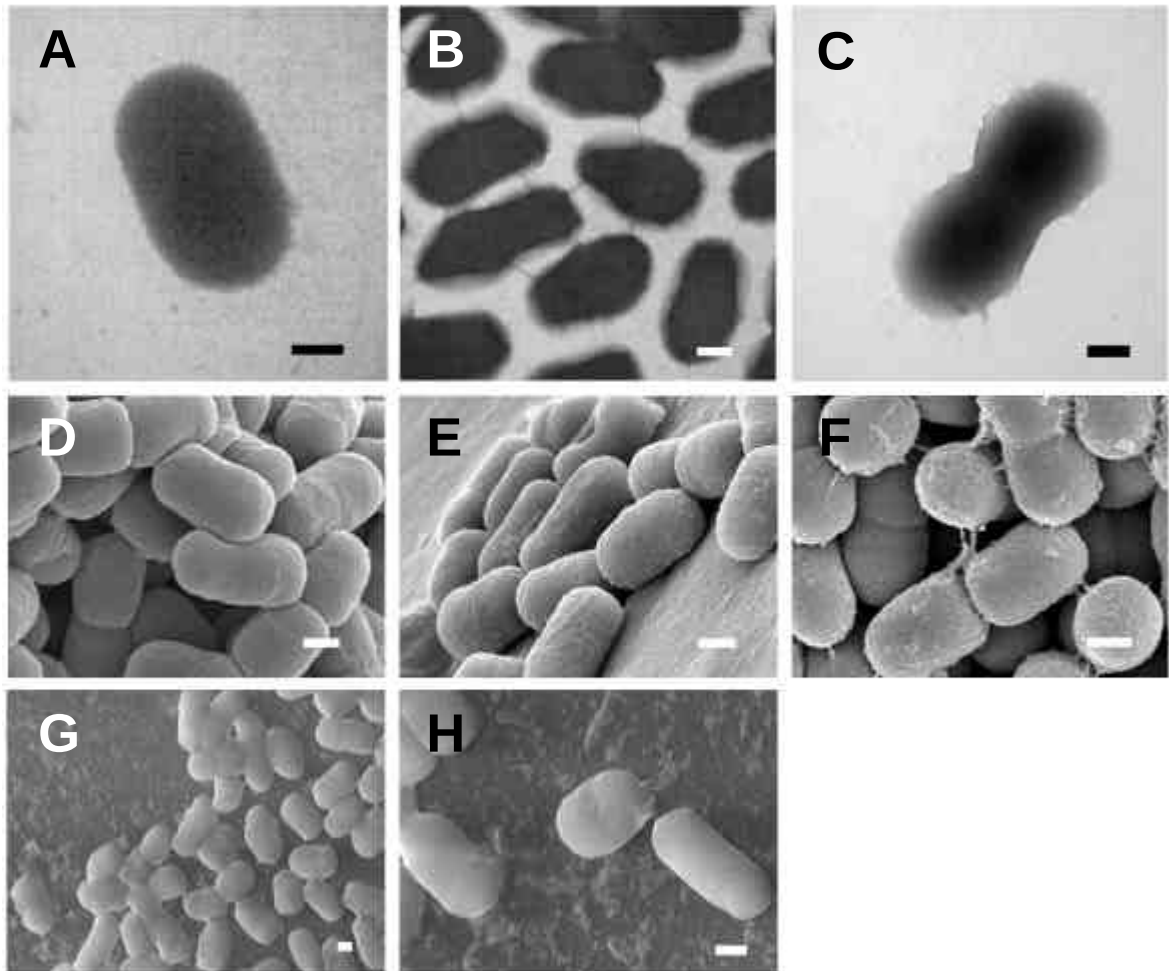


Fig. 3-12 Representative images of cell surface morphology of less-adhesive mutant, T1. Almost all of T1 cells had no appendage under TEM negative stained by phosphotungstic acid (**A**), however small number of T1 cells connected each other through short, thin appendages (**B**). T1 cells also revealed no appendage under TEM negative stained by ruthenium red (**C**). The planktonic cells (**D**) and sparsely adhered cells to polyurethane carrier (**E**) of T1 were observed under FE-SEM. The short appendages were shown at the small aggregate of T1 cells under FE-SEM (**F**). VP-FESEM shows the real nature of T1 cells adhering to the polyurethane carrier (**G, H**). bars = 300 nm.

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The observation under TEM and FE-SEM revealed that almost all of T1 cells have lost both the anchor and the pili-like appendages (Fig. 3-12 A, C, D, E). This proved that these appendages are necessary for Tol 5 to possess the strong adhesive ability. However, the short appendages which length is shorter than 200 nm were found in T1 by scrutinizing the micrographs, even though they were observed with a quite low frequency (Fig. 3-12 B, F). Because of its short and thin features, this appendage is suspected to be different from appendages found on WT cells but to contribute to the adhesiveness of the less-adhesion mutant T1 (Fig. 3-2). Note that even T1 was not less adhesive than adhesive *P. aeruginosa* PAO1 (Fig. 3-2). The observation of T1 cells under VP-FESEM was also consistent with the images obtained by FE-SEM and TEM, which indicated that T1 cells have lost any appendage (Fig. 3-12 G, H). However, short appendage could not be found by VP-FESEM because of less resolution of this instrument. Most probably, these results suggest that anchor and pili-like appendages are important “adhesins” of Tol 5 cells.

Among the less-adhesive mutant strains, T6 retains weak aggregativeness (Fig. 3-13 A). T6 cells adhered to the polyurethane carrier in the foam of single-layers of cells along with small cell cluster (Fig. 3-13 B). T6 cells adhere to polyurethane carrier, glass cover slip, and mica surface less than WT cells, but they are more adhesive than T1 cells (Fig. 3-13 C, D). This suggests that expression of the appendages is different on the T6 cells from on the T1

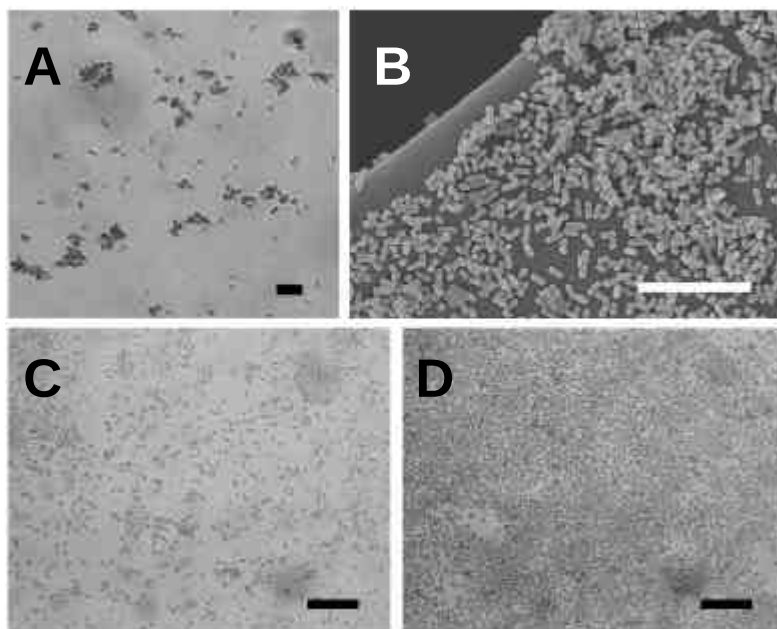


Fig. 3-13 (A) Cell suspension in the liquid phase of less-adhesive T6 mutant. (B) Adhesion to the polyurethane carrier of T6 cells at the stationary phase under FE-SEM. T6 cells were cultivated to the stationary phase in the presence of the glass cover slip (C) or mica (D). Bars = 10 μm.

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cells. The observation of T6 cells by TEM revealed two different types of cells. One is a little aggregated cell that has some straight appendages (Fig. 4-14 A), the other is suspended cell that has no appendage (Fig. 4-14 B). However, T6 cells were not stained under the ruthenium red stained (Fig. 3-14 C) while the cell body of both WT and T1 cells were negatively stained with ruthenium red (Fig. 3-4 G-I, 3-12 C). It suggests that LPS of T6 cells are not exposed to ruthenium red because of coating of an unknown compounds. The observation of T6 cells by FE-SEM revealed that T6 cells possess appendages, which seem morphologically different from the anchor of WT cells (Fig. 3-14 D-F). The appendages do not extend straight but twist without branching. They resemble the pili-like appendages of WT cells in length and morphology. The feature of the interaction between the appendages of T6 cells and a substratum was similar to the case of pili-like appendages of WT cells; the interaction extended over the large part of the length of the appendage (Fig. 3-14 B). Therefore, T6 cells seem unable to be tethered at a long distance from the substratum like WT cells. By these observations, the lost of the functional anchor may have deprived Tol 5 cells of highly adhesive property. However, the observation of T6 cells under VP-FESEM (Fig. 3-14 C) showed a little different feature of the cell appendages, which is quite long compared with the images by FE-SEM (Fig. 3-14 B). This appendage was easily removed by the washing of 50% ethanol solution, while the appendages of WT were hardly removed by same treatment (Fig. 3-14 D).

In addition, the observation of T2, T3, and T4 cells by FE-SEM revealed that these cells have also lost the anchor and pili-like appendage, supporting that the appendages are necessary for Tol 5 to adhere to the substratum (Fig. 3-15 A-C). Indeed, the appendages were seen on T5 cells, which is a control mutant that retained adhesive property (Fig. 3-15 D). All these results strongly suggest that the anchor and pili-like appendages on the cell surface are responsible for the strong adhesive property of Tol 5 (Table 3-3).

Table 3-4 summarized the cell surface characteristics of WT and the less-adhesive mutants. T1 cells retained the high adsorptivity to hexadecane droplets, but showed a small contact angle of a water droplet compared with WT cells, implying a decreased water-repellent property by transposon mutagenesis. According to the value of ζ potential, the net negative charge of the T1 cell surface was decreased, suggesting that negatively charged structures on the cell surface were deleted by the mutagenesis. T6 cells showed a considerable hydrophilic feature according to both results from MATH test and contact angle measurement, however the value of ζ potential was retained. These results suggest that the appendages on the cell surface affect cell surface characteristics such as hydrophobicity and surface electrical charge.

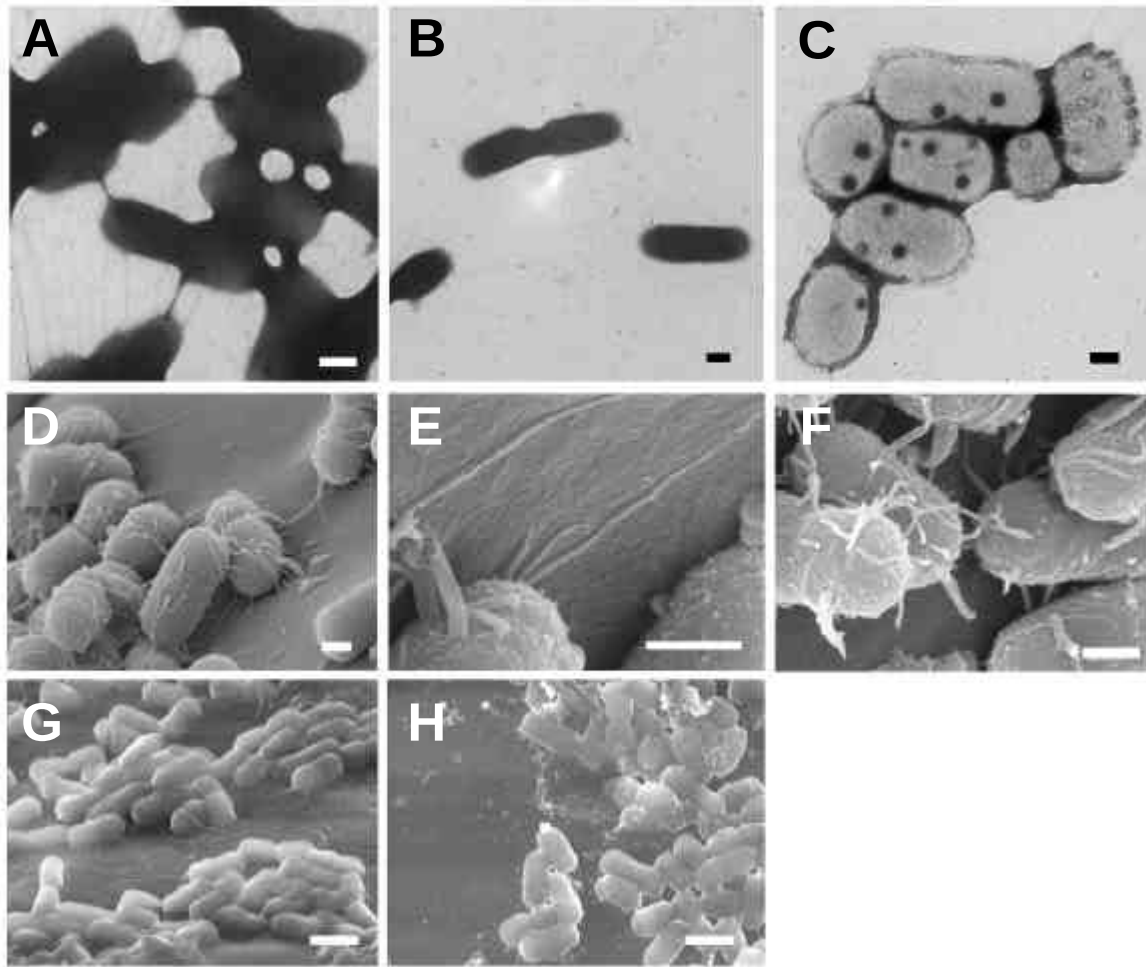


Fig. 3-14 Representative images of cell surface morphology of less-adhesive mutant, T6. Aggregated T6 cells had some appendages under TEM negative stained by phosphotungstic acid (A). However, almost all of suspended T6 cells had no appendage (B). T6 cells were not stained by ruthenium red because of the coating of an unknown compound (C). Under FE-SEM observation, the cells adhered to polyurethane carrier with cell surface appendages but had no anchor (D - F). The close-up image of the vicinity of glued region of T6 shows the appendage crawled onto the substratum (E). bars = 300 nm.

VP-FESEM showed the real nature of T6 cells adhered to the polyurethane carrier (G). A carrier was washed by 50% ethanol solution to remove EPS (H). bars = 1 μ m.

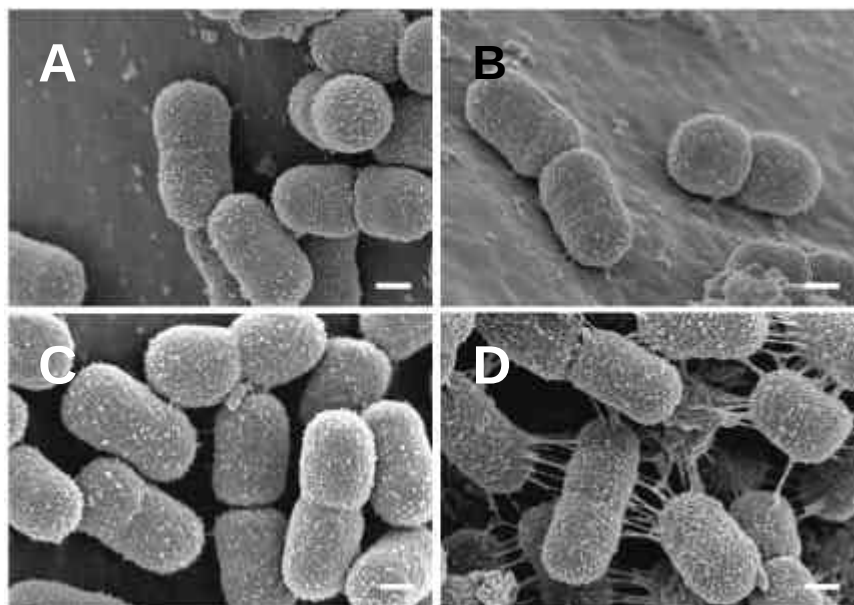


Fig. 3-15 Representative images of the cell-surface morphology of the mutant strains obtained by FE-SEM. The less-adhesive T2 (A), T3 (B), and T4 (C) cells. The T5 cells retained adhesiveness (D). Bars = 300 nm

Table 3-4 Analysis of cell surface hydrophobicity and ζ potential of mutants

Bacterial strain	MATH test (%)*	Contact angle (°)	ζ potential (mV) [†]
WT	91.8	88.0	– 13.23
T1	87.1	42.8	– 3.17
T6	21.3	43.2	– 12.90

* Hydrophobicity (%) = $(OD_{660\text{-initial}} - OD_{660\text{-after extraction}}) / OD_{660\text{-initial}} \times 100$

[†] ζ potential were measured in 9.4 mM PBS (I = 0.014 M) at pH 7.4

3-3-8. Cell surface protein of Tol 5 cells

A WT-specific surface protein was detected through biotinylation-SDS analysis (Fig. 3-16). In this method, only cell-surface proteins exposed to the liquid phase are labeled with biotin. After total proteins from WT, T1, and T6 were separated by SDS-PAGE, the biotinylated proteins were detected by Western blotting using streptavidin-conjugated horseradish peroxidase. The blot showed a WT-specific band of 18 kDa that was not present in T1, and T6. It is likely that this surface protein is one of the main components of the appendages. The image also showed a mutant-specific band of 26 kDa, which is likely concerned with the insertion of the vector to the WT cell.

Two-dimensional gel electrophoresis also showed the difference between the total protein profiles of WT and T1 cells. The image showed a WT-specific band over 150 kDa that was not present in T1 (Fig. 3-17). This protein is probably a component of the appendage. Mutant-specific bands were also identified in the image, one of which had the same size as that of the protein observed in figure 3-16. It is necessary to analyze the protein components of the appendages in further work, because the proteinaceous appendages usually have many components.

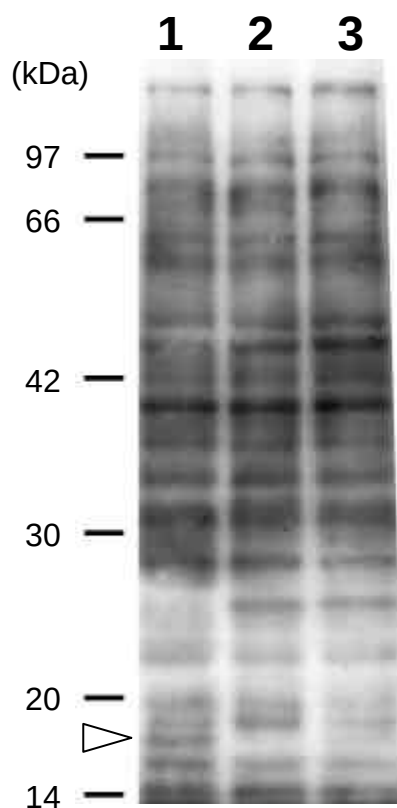


Fig. 3-16 Cell-surface proteins labeled with biotin and detected by Western blotting using streptavidin-conjugated horseradish peroxidase. After the biotinylation of surface proteins, total cell proteins of WT (lane 1), T1 (lane 2), and T6 (lane 3) were subjected to SDS-PAGE and electroblotted onto a PVDF membrane. The open triangle indicates a WT-specific surface protein at 18 kDa.

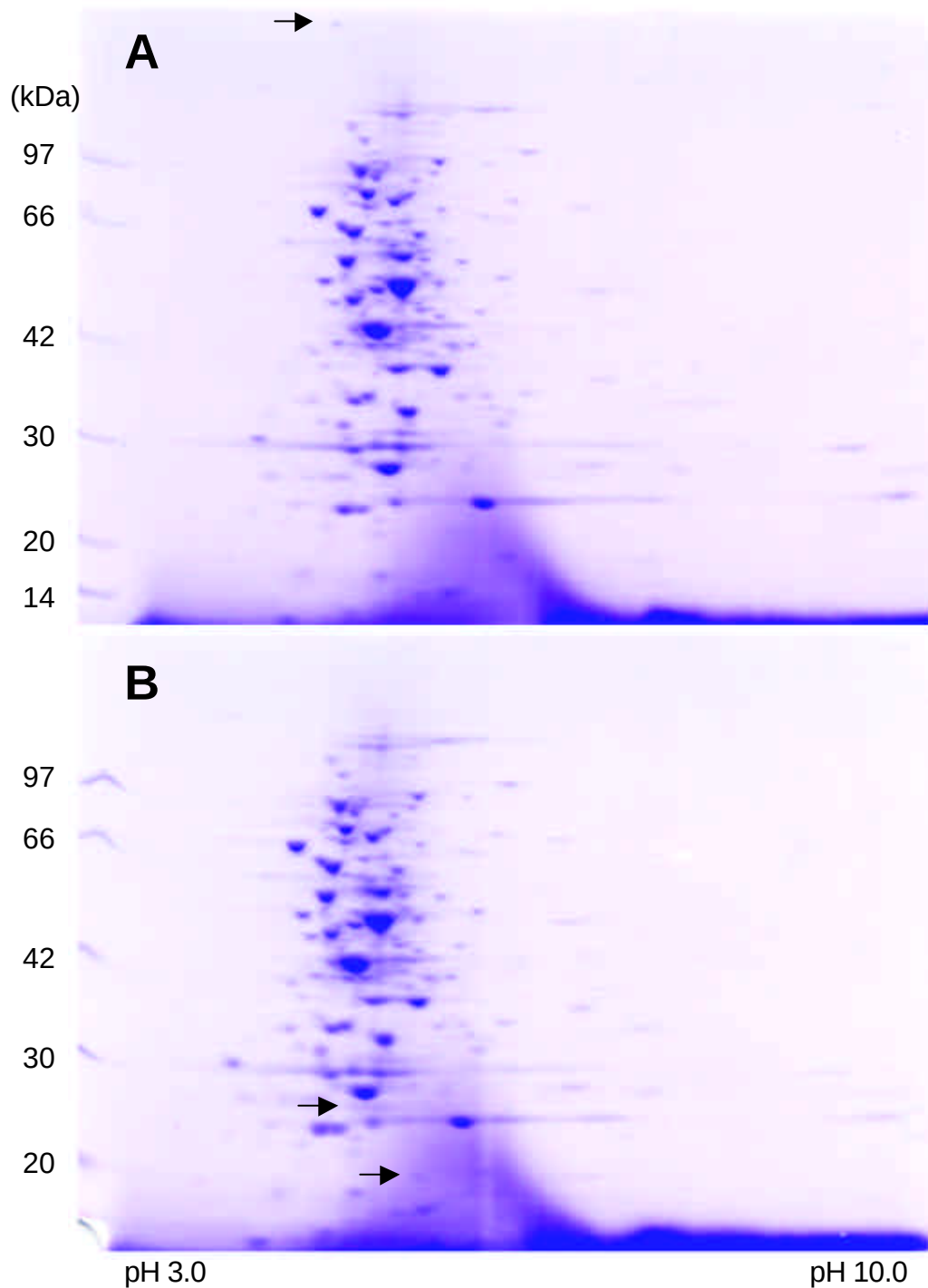


Fig. 3-17 Two-dimensional images of crude protein extracts of WT cells (**A**) and T1 cells (**B**). The crude protein extracts (100 μ g) were separated on pH 3 to 10 by isoelectric focusing electrophoresis, followed by SDS polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue. The arrows indicate the specific proteins.

3-3-9. AFM observation of cell surface appendages

Recently, the new technique of atomic force microscopy (AFM) is supposed to show nearly 'real' images of bacterial cell surfaces and appendages, because there is no need for sample fixation to visualize a bacterial cell at high resolution [25, 39]. At the AFM observation, the tip of cantilever taps the cell surface, thereby the exogenous destruction is caused by the mechanical press. Moreover an excluded volume through the cantilever is responsible for the visualization of artifact structure in case of the observation of considerably

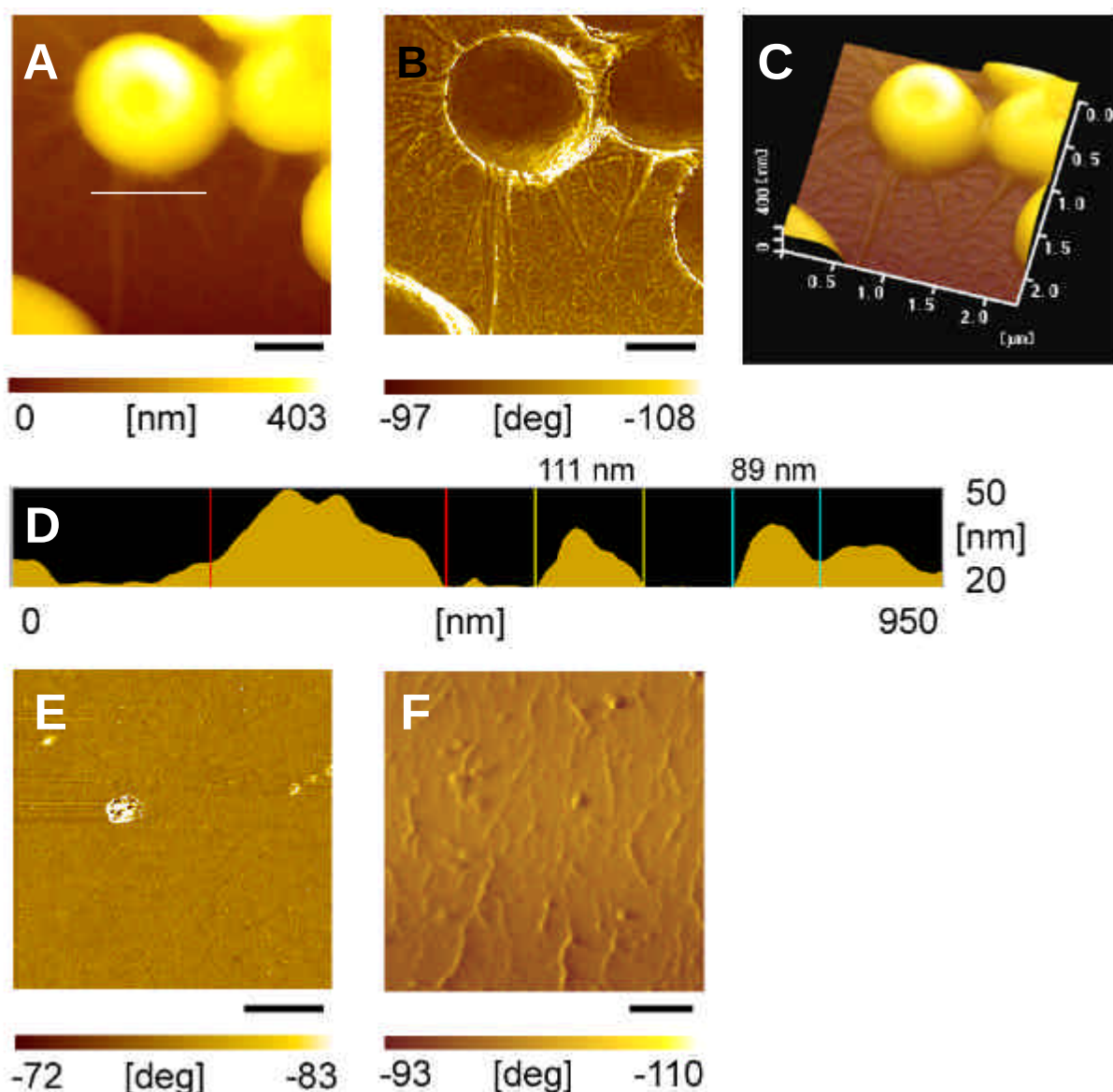


Fig. 3-18 AFM images of the appendages of WT cells on a hydrophobic glass surface by using AC mode in air; (A) topography image, (B) phase image, (C) 3D image. White line indicates the cut position of the horizontal cross sections (D). A phase image of the untreated hydrophobic glass (E) was compared to the image of the glass immersed in the supernatant of the saline used to wash WT cell (F). All bars = 500 nm.

III. Cell surface appendages

high and steep specimen such like bacterial cell body. WT cells with the appendages were observed by AC non-contact mode imaging of AFM (Fig. 3-18). The pili-like appendages that crawled on the hydrophobic glass surface were observed, but the existence of the anchor-like appendage was not revealed (Fig. 3-18 B). The height of pili-like appendages was approximately 20 nm, though the specimen might be compressed by the cantilever (Fig. 3-18 D). In the phase image, netted pattern was seen on the background of a glass surface (Fig. 3-18 B). To identify what produced the netted pattern of the background, the glass slip immersed in the saline where bacterial cells were suspended and removed was also observed. On this glass surface, adhesion of fibrous materials was seen while the intact hydrophobic glass has flat surface (Fig. 3-18 E, F). This suggests that the netted pattern were attributed to some cell surface structure cut off by shear stress from washing process. The AFM images of the T1 cell showed the deletion of any appendages and the netted pattern on the glass surface (Fig. 3-19). Theses results were consistent with these of FE-SEM observation.

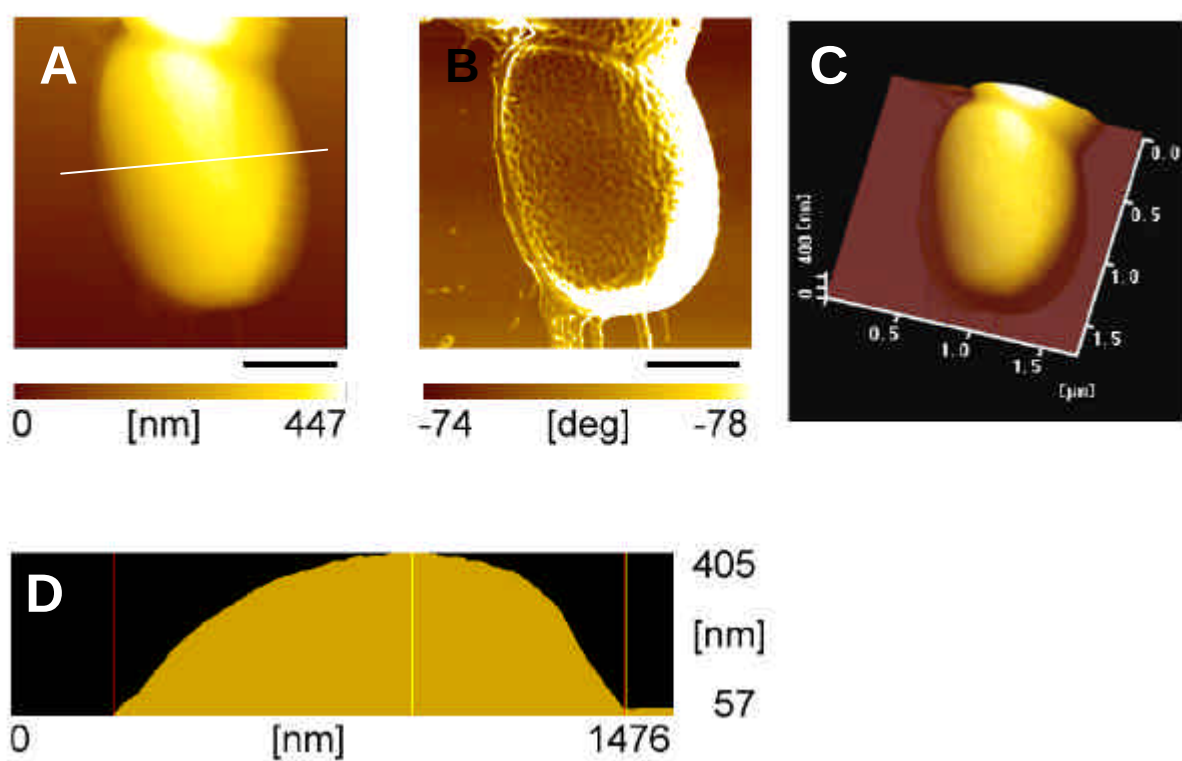


Fig. 3-19 AFM images of the appendages of T1 cells on a hydrophobic glass surface by using AC mode in air; (A) topography image, (B) phase image, (C) 3D image. White line indicates the cut position of the horizontal cross sections (D). All bars = 500 nm.

3-4. Summary

Acinetobacter *genospecies* Tol 5 shows extreme adhesive properties as compared with other adhesive bacteria such as *Pseudomonas aeruginosa* PAO1. A unique anchor-like appendage as well as pili-like appendage was discovered on Tol 5 cells using field-emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM). The anchor-like appendage extends straight from the cells to the substratum without branching and its end interacts with the substratum. The bacterial cells use it to tether themselves to the substratum like a balloon on a string at distances of several hundreds of nanometers. This striking feature of the anchor was confirmed to be intact by observation under a variable-pressure FE-SEM equipped with an environmental secondary electron detector, which dispenses with the sample preparation needed for conventional SEM observation. A less-adhesive mutant, T1, has lost these appendages, while another less-adhesive mutant, T6, has appendages that appear morphologically different from the pili-like appendages of the wild-type strain. Protein was found to be an important component of the anchor by trypsin treatment. In addition, biotinylation-SDS analysis of the cell-surface proteins and two-dimensional gel electrophoresis of the crude proteins of the WT and mutant strains revealed a band specific to the former.

Chapter IV

Adhesion dynamics through anchor-type appendage

4-1. Introduction

Biofilms are sessile bacterial communities formed on solid surfaces. Biofilm, which consists of the multi-layer bacterial cluster, is sometimes harmful to both human life and industrial processes. Biofilm formation process of *Pseudomonas aeruginosa* has been studied by many researchers, because this strain forms biofilms on various surfaces, causing nosocomial infections in immunocompromised patients and individuals with severe burns [6]. Fig. 4-1 shows four stages of biofilm development of *P. aeruginosa*: (I) reversible attachment, (II) irreversible attachment, (III) microcolony formation, and (IV) matured biofilm [76]. In this process, first two steps are important to control the bacterial adhesion, which is caused by physicochemical interactions and bridging effects of appendages. O'toole *et al.* showed that

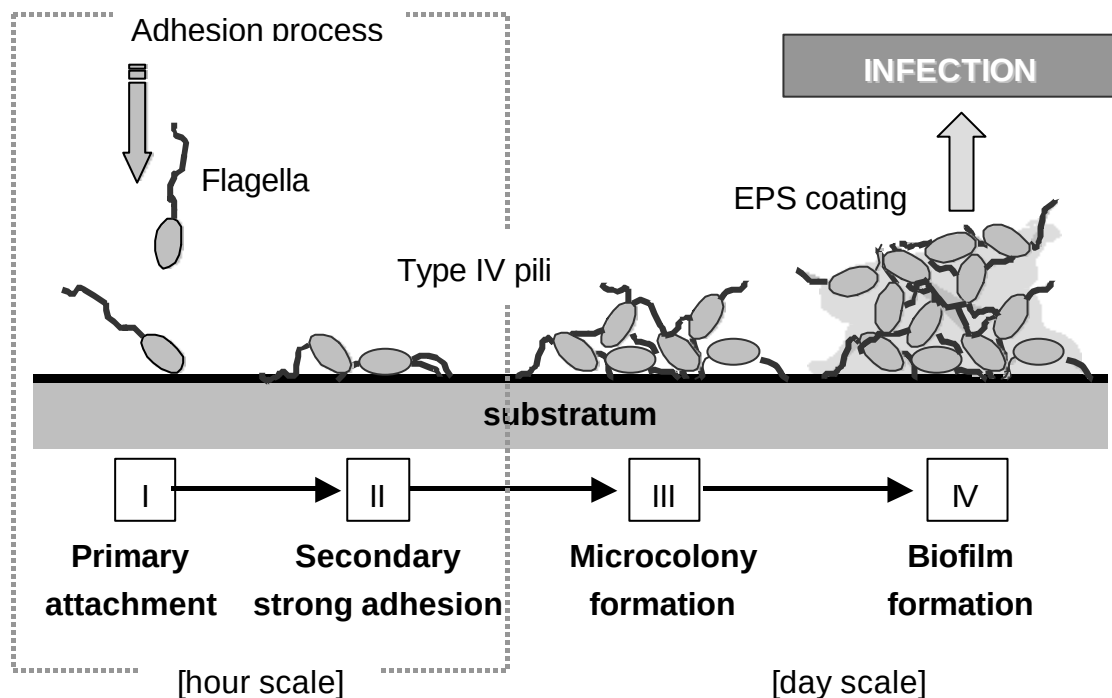


Fig. 4-1 Typical biofilm formation process of *Pseudomonas aeruginosa*. The infection by this strain is caused by the mature biofilm (phase IV). Adhesion process consists of two stages of reversible attachment (phase I) and subsequent irreversible adhesion (phase II).

both flagellar-mediated motility for the first attachment and the type IV pili for the microcolony formation are important for bacterial adhesion [59].

The standard model of bacterial adhesion was developed from Derjaguin-Landau-Verwey-Overbeek (DLVO) theory [50], which describes the total interaction energy of a colloidal particle with a surface as a summation of van der Waals and Coulomb interactions (Fig. 4-2) [50, 89]. On the basis of the DLVO theory, bacterial adhesion is considered 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) [3, 7, 50, 94] (Fig. 4-3). According to the DLVO theory, this transition is considered to be related to movement from a secondary to a primary energy minimum [50] (Fig. 4-2). Between these two energy minima, there is an energy barrier that bacterial cells cannot cross by swimming or Brownian motion.

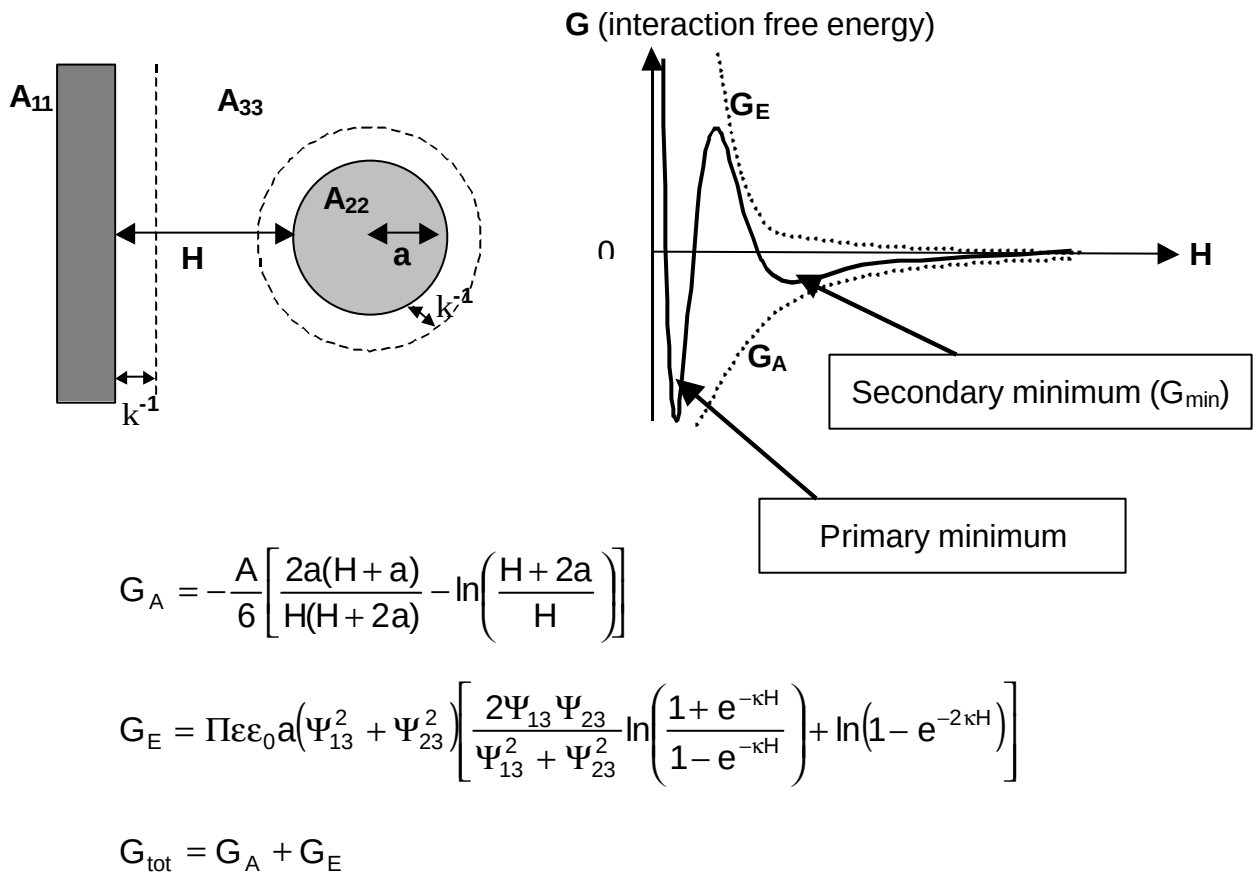


Fig. 4-2 Long-range interactions according to the DLVO theory. G_A , free energy of the van der Waals forces; G_E , free energy of the electrostatic interaction; G_E , total interaction Gibbs energy; a , radius of the particle; H , separation distance; $\hat{e}^{-1}$, double layer thickness; A , net Hamaker constant of the system (A_{11} of the surface, A_{22} of the particle, A_{33} of the third phase); $\hat{a}$, dielectric constant of the medium; $\hat{a}_0$, permittivity of free space; $\emptyset$, electric potential at the surface ($\emptyset_{13}$ between surface and medium, $\emptyset_{23}$ between particle and medium). [50, 89]

Cell appendages, such as pili and flagella, or exopolymeric substances (EPS) can pierce this energy barrier, reach the primary energy minimum, which is closer to the substratum than the secondary one, and function as a bridge for irreversible adhesion in phase two [3, 7]. However, the height of the energy barrier [56, 64], the depth of the secondary energy minimum related to the reversibility of adhesion [56, 94], and the real force that holds cells reversibly [94] remain controversial, although extended models of the DLVO theory have been proposed [7]. In all cases, adhesion mechanisms are closely related to the distance between the cell and a substratum, and bacterial adhesion has been proposed as a phenomenon initiated by cells within 50 nm, but more typically within 10 nm, of a substratum [3, 7, 32, 50, 56, 67, 70, 89, 93, 94].

Electrostatic force plays an important role for the bacterial adhesion in the DLVO theory. Cell surfaces of most bacteria have a net negative charge as well as surfaces of almost all of abiotic materials in the electrolyte [67, 89, 97]. The repulsive electrostatic energy is caused by the overlap of the electrical double layer, the thickness of which is depended on the net negative charge of surfaces and the ionic strength of electrolyte. This repulsive energy can be lowered by increasing the ionic strength of suspension medium because of the compression of the electrical double layer. Depending on the electrolyte concentration, three different interaction energy profiles can be distinguished as calculated from the DLVO theory (Fig. 4-4): (i) at low ionic strength, when the double layers on both cell and solid surfaces are extensive, the net interaction between them is repulsive over the separation distance, and the

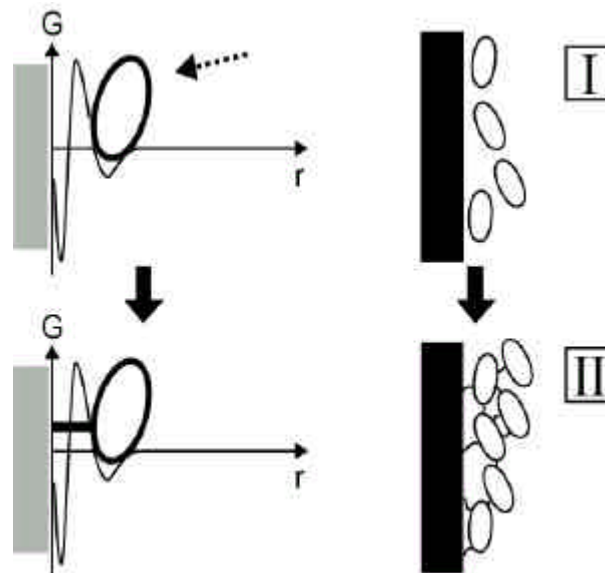


Fig. 4-3 Two step adhesion model based on DLVO theory. Total interaction energy (G) at the distance (r) from the substratum is calculated. An initial, instantaneous, and reversible physical phase (I) and a time-dependent and irreversible bridging phase by the adhesin (II) were first proposed by Marshall *et al.* [50]

two surfaces may not come into contact; (ii) at moderate ionic strength, a shallow secondary energy minimum may be formed at some separation distance (typically 5 to 15 nm), where the cell may be attached in a reversible manner; (iii) at high ionic strength, the net interaction is attractive over the separation distance and results in a strong, basically irreversible adhesion [67, 89, 97] (Fig. 4-4). Consequently, bacterial cells are difficult to adhere to solid surfaces in a liquid with lower ionic strength, if the DLVO theory is effective to explain bacterial adhesion process.

In chapter III, the anchor-like appendage was found on a cell of strongly adhesive *Acinetobacter genospecies* Tol 5. The cells use only the very end of the anchor to tether them to the substratum like a balloon on a string at distances of several hundreds of nanometers. By using this especial appendage, Tol 5 cells can be tethered at a distance more than 500 nm from a substratum, although the bacterial cells need to be captured within 50 nm in classical two-step adhesion process calculated by DLVO theory.

To prove the new mechanism that differs from the previous models based on the two-phase process, the adhesion dynamics of Tol 5 cells were analyzed in this chapter. At first, the adhesion rate of Tol 5 cells and reversibility of adhered cells were analyzed. Then, the effect of ionic strength on bacterial adhesion of Tol 5 was investigated. And other factors that may affect the bacterial adhesion were studied.

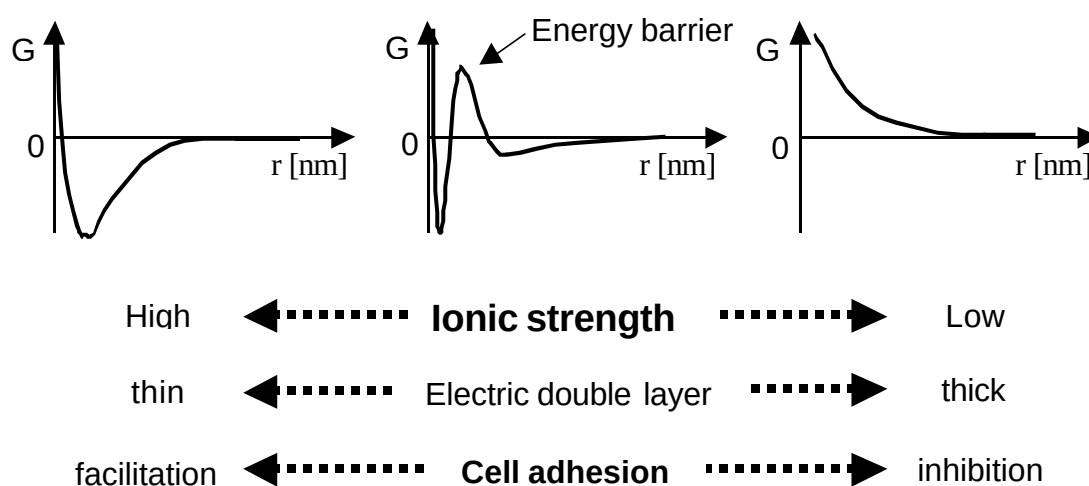


Fig. 4-4 Accessibility of bacterial cell to a substratum for adhesion affected by the ionic strength of the medium, assumed from the DLVO theory. Low ionic strength of the medium makes the energy barrier higher, resulting in the inhibition of bacterial adhesion.

4-2. Materials and methods

4-2-1. Adhesion test and detachment test

Adhesion tests were carried out by the same procedure as described in chapter III (see 3-2-4). To analyze adhesion dynamics, the culture broth was sampled with time (from 30 s to 1h). In order to investigate the effect of ionic strength, adhesion test was conducted in the electrolyte with a wide variety of ionic strengths. The cells were washed with deionized water, followed by immersed in the gradual series of diluted solution of BS medium ($I = 0.34 \text{ mM}$). After addition of polyurethane carrier to the cell suspension, the flasks were incubated at 28°C with shaking at 115 rpm for 30 min.

Detachment tests were carried out to determine the reversibility of cell adhesion. At 30 s during the adhesion test, the polyurethane carriers, to which the bacterial cells were partially adhered, were collected. 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 number, the cell fraction remaining on the polyurethane was calculated as follows:

Here, the number of total cells in a flask corresponds to the number of the cells that adhered

$$\text{Cell fraction on the carrier (\%)} = \left(1 - \frac{\text{the number of detached cells from the carrier}}{\text{the number of total cells in a flask}} \right) \times 100$$

during 30 s of incubation in the adhesion test.

4-2-2. Separation of aggregated cell from suspend cell of Tol 5

Tol 5 cells had been grown to stationary phase with toluene as the sole carbon source without the polyurethane carriers. Aggregated cells and suspend cells were separated through setting by standing for 30 min. Each cell fractions were washed with deionized water and provided to the adhesion test as a function of ionic strength and FE-SEM observation.

4-3. Results and discussion

4-3-1. Irreversible adhesion of wild-type cells

In order to determine the reversibility of the anchor-mediated adhesion of Tol 5 cells to the polyurethane carrier, adhesion and detachment tests were carried out. Here, the reversible state means that adhered cells can detach from a substratum by Brownian motion or inherent cellular motility, and revert to the planktonic state. First, 8.3×10^9 WT, T1 or T6 cells grown on toluene were incubated at room temperature in 20 ml of fresh BS medium in the presence of pieces of polyurethane carrier with a total surface area of 6,400 mm² (adhesion test, Fig. 4-5). The half number of Tol 5 (WT) cells in the medium adhered to the polyurethane foam within 30 sec, and 90% of WT cells adhered to the carrier within 5 min. Eventually almost all of WT cells adhered to the carrier, while adhesion ratio of T1 cells and T6 cells were 16% and 44%, respectively. These results suggest that Tol 5 cells are able to adhere to the solid surface instantaneously by bridging between the cell body and a substratum through the anchor and/or pili-like appendages.

After 30 s of incubation at 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 (detachment test). During this

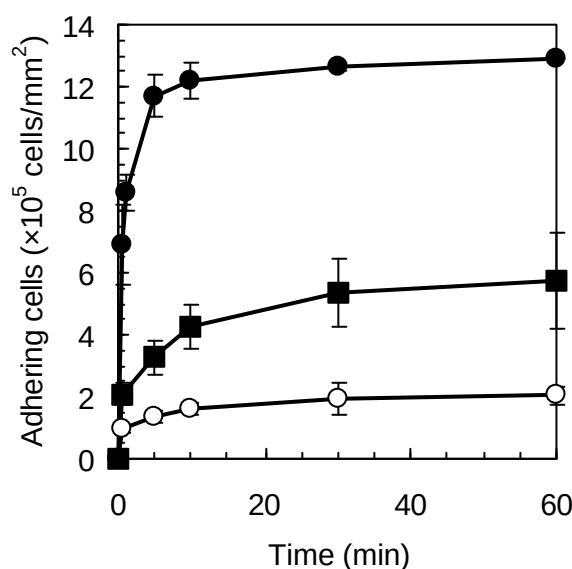


Fig. 4-5 Specific numbers of adhering cells during adhesion tests of WT (solid circle) and a less-adhesive mutant, T1 (open circle) and T6 (solid square), of *A. genospecies* Tol 5. The pre-grown cells (8.3×10^9 cells/ml) were transferred to 20 ml of fresh BS medium lacking a carbon source and incubated in the presence of the polyurethane carrier with a total surface area of 6,400 mm². The decrease in OD₆₆₀ during incubation was measured to calculate the number of adhering cells (n=5).

detachment test, a proportion of the adhered cells of T1 and T6 detached from the polyurethane carriers (Fig. 4-6). Finally, the fractions of detached T1 and T6 cells almost reached a plateau at a level of 45% and 33% respectively, which is considered to represent the adsorption-desorption equilibrium and the transition from the reversible to the irreversible state, as this transition is expected to continue during the incubation of the detachment test. This implies that the T1 and T6 cells that adhered during the 30 s of incubation in the adhesion test were still attached in a reversible state. By contrast, WT cells did not detach, which implies that WT cells 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 WT cells would have detached from the polyurethane surface into the fresh medium by Brownian motion of the cells. These results suggest that the anchor mediates instantaneous, irreversible adhesion of Tol 5 cells to a solid surface.

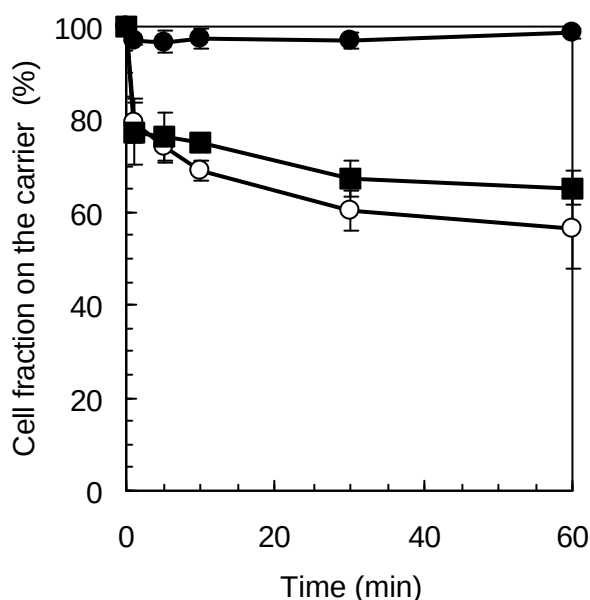


Fig. 4-6 Fraction of cells remaining on the polyurethane carrier during detachment tests of WT (solid circle), T1 mutant (open circle), and T6 mutant (solid square) of *A. genospecies* Tol 5. Polyurethane carriers to which cells had partially adhered during 30 s of incubation in the adhesion test were collected and transferred to 20 ml of fresh water in a flask for further incubation. Cell fractions on the carrier were calculated from the detached cell fractions obtained by dividing the numbers of cells released by the number of cells that initially adhered to the carrier (n=5).

4-3-2. Proposal of one-step adhesion mechanism through anchor

Adhesion to polyurethane foam was quantitatively estimated by the DLVO theory in terms of electrostatic interaction due to the overlap of the electrical double layers at the charged surfaces [50, 89]. The influence of the electrostatic interactions was assessed by varying the ionic strength, which is the parameter to be easily controlled in an experiment and indeed, many researchers have studied their effects on bacterial adhesion [20, 21, 24, 30, 51, 70, 71, 90, 93, 98].

Fig. 4-7 showed the change of adhesiveness of WT and T1 cells to the polyurethane carrier as a function of ionic strength. WT cells retained adhesiveness with decrease in the ionic strength (Fig. 4-7, A). This suggests that the electrostatic repulsive interaction does not affect the strong adhesion of WT cells, and therefore this suggests that WT cells do not follow expected trends predicted by the DLVO theory. In contrast, adhesiveness of T1 cells increased from almost 0 to 2.5×10^5 cells/mm² when the ionic strength increased from 0 mM to 340 mM (equal to BS salt) (Fig. 4-7 B). This suggests that electrostatic repulsive interaction at low ionic strengths crucially affects the adhesion process (see Fig. 4-4), and therefore T1 cells, which have lost the appendages, adhered to solid surfaces through the two-step mechanism presumed by the DLVO theory [20, 24, 30, 70, 71, 90, 98].

In the two-step adhesion process, initial adhesion is caused at the distance of the secondary energy minimum ($G_{\min}$) (Fig. 4-2). Hence, both the transport and retaining of the cells onto the secondary energy minimum are necessary for bacterial adhesion. The transport of bacteria towards the surface can occur by different modes: diffusion by Brownian motion (average displacement of 40 μ m/h), convective transport due to liquid flow (several orders of magnitude faster than diffusion), and active bacterial movement by chemotactic activity (10 – 20 μ m/s) [56, 67]. After the cells are transported close to the adhesive surface, adhesion takes place under the control of physicochemical interactions and shear forces [72]. It has been considered that the time during which a bacterium remains reversibly at the secondary energy minimum is proportional to $\exp[|G_{\min}|/kT]$, thereby the retaining time become longer with increase in the ionic strength because $G_{\min}$ become deeper [74].

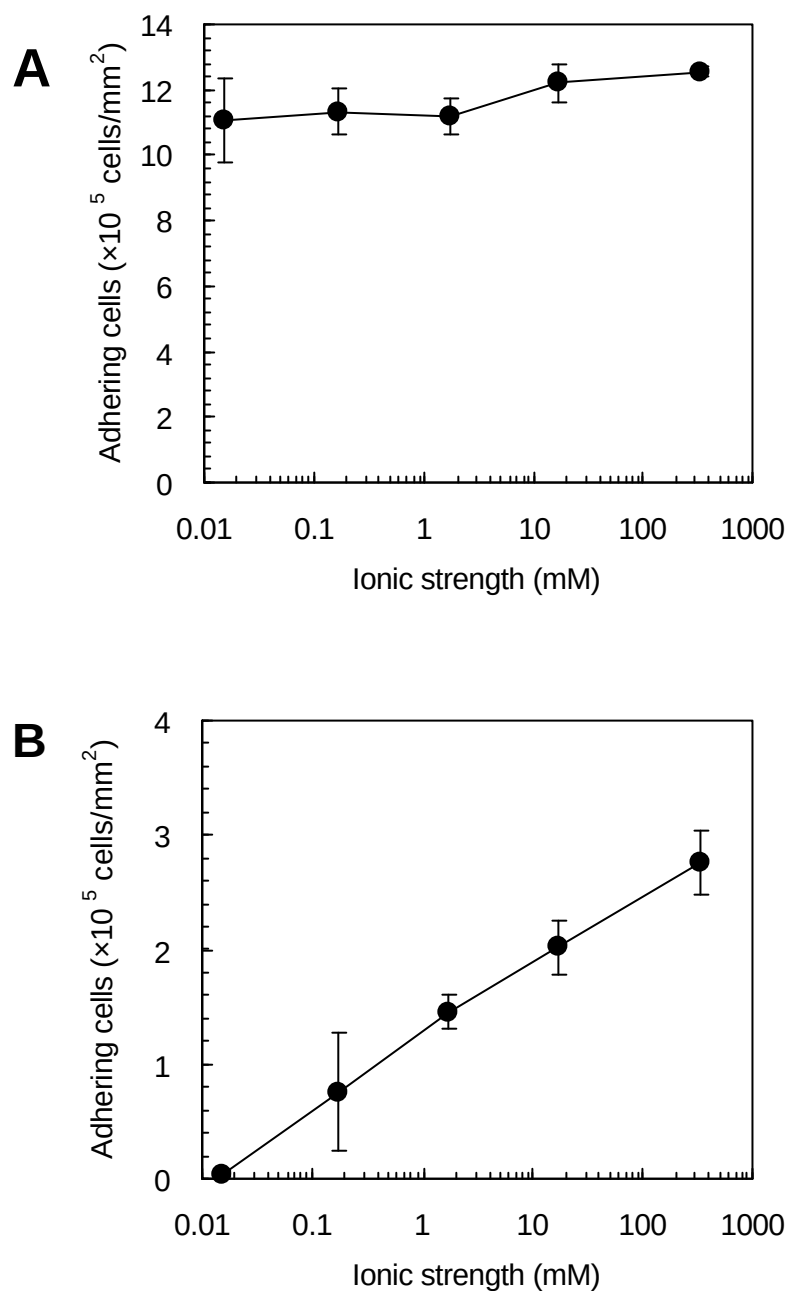


Fig.4-7 Dependency of adhesiveness of bacterial cells to the polyurethane carrier as a function of ionic strength. WT cells (A) and T1 cells (B) were incubated with the polyurethane carrier for 30 min at 28°C, 115 rpm, followed by determination of the cell adhered to the carrier (n=5).

If the T1 cells are postulated to adhere to solid surfaces through the two-step mechanism as mentioned above (Fig. 4-7 B), the cells should be transported to the secondary energy minimum and retained there until they attain the irreversible adhesion. FE-SEM image of adhesion of T1 cells to the polyurethane surface clearly show the cells accumulating on cracks or grooves on the surfaces (Fig. 4-8 A). Thus, adhesion preferably starts at locations where the cells are sheltered from shear forces and increase in the chance of staying at the secondary energy minimum until the second step of the adhesion process. In this step, T1 cells were revealed to attain irreversible and strong adhesion with the short appendages described in the previous chapter (Fig. 3-12 C, F). In the cracks or grooves, T1 cells adhering to the substratum with small aggregate by using the short appendages were found under FE-SEM (Fig. 4-8 B). Fibrous appendages have considerably smaller radii than the whole cell. Since the electrostatic repulsion energy depends more strongly on the particle radius than the van der Waals attraction (i.e. G_{tot} is smaller for smaller particle radii), appendages can reach the primary energy minimum regardless of the energy barrier for the whole cell. As a result, they will bridge the gap between surfaces and a bacterial cell [50, 74, 89].

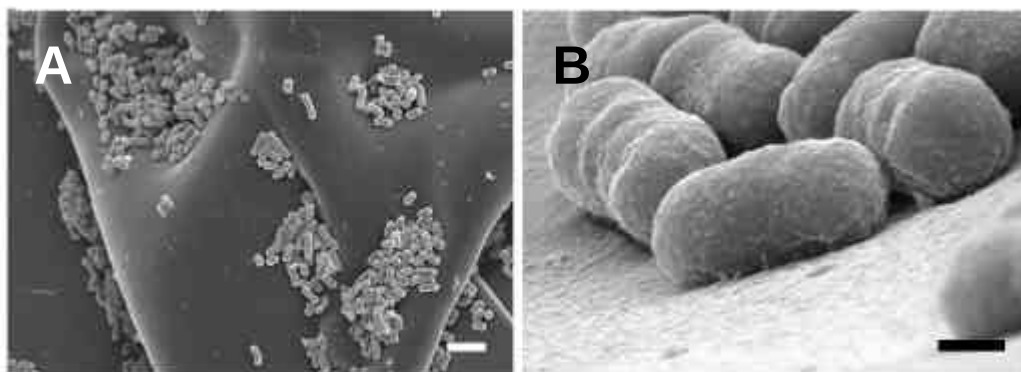


Fig. 4-8 Specific images of T1 (A) cells adhering to the surface irregularities such as cracks and grooves, on polyurethane foam observed by using FE-SEM. bars = 3 μm . (B) The close-up images of T1 cells showed short appendages. bars = 300 nm.

Indeed, many researchers have shown that cell appendages are involved in bacterial adhesion [3, 5, 18, 23, 39, 59, 65, 67]. Both proteinaceous appendages and EPS are considered to be the main factors responsible for tenacious adhesion in phase two of the adhesion process. For example, *E. coli* first attaches to the substratum reversibly through flagella, and subsequently attains irreversible adhesion with type I pili [65, 94]. *P. aeruginosa* also follows the two-phase adhesion mechanism by using both flagella for motility and type IV pili for fixation [5, 23, 59]. Cells of *Flexibacter aurantiacus* CW7 are connected close to the substratum by EPS, which forms a wedge-like mass [49]. *Staphylococcus epidermidis* adheres to glassware through polysaccharide intercellular adhesin (PIA) after first forming a reversible attachment [27, 45]. The appendages on T1 cells seem quite short but still enough to function as the bridge because the distance from the substratum to the cell body at the secondary energy minimum is several tens nanometers.

As explained above, in the two-phase adhesion model, cells must be captured near a substratum before appendages can penetrate the energy barrier and interact with the substratum directly [50, 67, 89]. The reported distances for cell capture are conflicting and depend on the theory: for example, capture must occur within 10 nm of the secondary energy minimum in the DLVO theory [7], or 20 nm in the hydrodynamic effect [94]. The importance of motility caused by flagella and pili for cells to approach substratum has been also reported. The research group of Kolter has shown that flagella-mediated motility is required for the adhesion of *P. aeruginosa* [59] and *E. coli* [65]. Morisaki *et al.* have shown that there is a correlation between the attachment rate and the speed of swimming that is mediated by flagella and controlled by the Na^+ and K^+ concentration in *Vibrio alginolyticus*, *Alcaligenes* sp. and *Alteromonas* sp. [38, 56].

However, *A. genospecies* Tol 5 does not have motility (Fig. 3-9), that is, the marked adhesiveness of this strain is independent of motility. The Tol 5 cells (WT) can be tethered

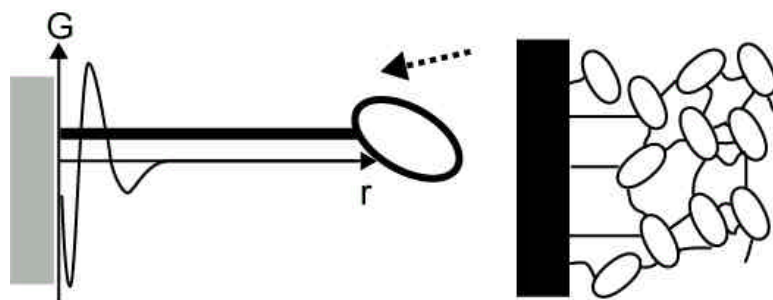


Fig. 4-9 Novel adhesion mechanism of WT cells through anchor. Total interaction energy (G) at the distance (r) from the substratum is calculated by DLVO theory. WT cells are tethered to the substratum by anchor from a long distance, hence the height of energy barrier has no correlation with bacterial adhesion.

more than 500 nm from a substratum by the anchors (Fig. 3-6 B, C). At this distance, cells are considered to be in a planktonic state that does not experience a force directing them to the substratum [94]. The finding of anchor-assisted adhesion suggests that Tol 5 attains irreversible adhesion from a planktonic state in a one-step process without passing through the reversibly captured state near a substratum (Fig. 4-9). In other words, the long distance connection assisted by the anchor seems to dispense with the need for the cell body to approach a substratum for adhesion. This hypothesis is also supported by the observation that Tol 5 cells attained irreversible adhesion instantaneously, that is, within 30 s (Fig. 4-5, 4-6). Hence, adhesion of WT cells of *A. genospecies* Tol 5 to solid surfaces does not follow the DLVO theory.

As seen in Fig. 3-3, the strong adhesion of *A. genospecies* Tol 5 is due to co-adhesion, which causes the formation of multi-layer cell clusters. The anchor not only tethers the cell to a substratum but also mediates cell-to-cell connections (Fig. 3-6 A, B). This type of connection is considered to be important for forming the multi-layer cell clusters.

The adhesion of the Tol 5 (WT) cells to the substratum is mediated by the connection between the tip of the anchor and a substratum (Fig. 3-6 B). When bacteria or its appendages directly contact with the surface, the interfacial free energies (G) or interfacial tensions ($\tilde{\alpha}$) between interacting surfaces can be used to describe the change in the interfacial excess Gibbs energy upon adhesion (ΔG_{adh}) (Fig. 4-10). When ΔG_{adh} is negative, adhesion is thermodynamically favored, and will proceed spontaneously [1, 7, 89]. This interaction is irreversible and much stronger than adhesion in the secondary minimum. In another approach, surface free energies are separated into long-range apolar or Lifshitz-van der Waals interactions ($\tilde{\alpha}^{LW}$) and short-range polar or Lewis acid-base interactions (hydrogen bond, $\tilde{\alpha}^{AB}$)

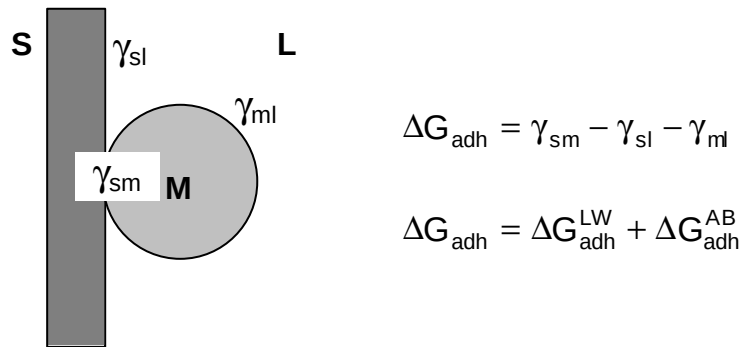


Fig. 4-10 Schematic representation of short-range interactions. The microorganism or its appendage (M) makes direct contact with the surface (S) forming new interface from a liquid suspension (L). γ , interfacial tensions; ΔG_{adh} , interfacial interaction energy; ΔG_{adh}^{LW} , Lifshitz-van der Waals interaction energy; ΔG_{adh}^{AB} , Lewis acid-base interaction energy. [89, 91]

[7, 91]. This enables to formulate a surface thermodynamic treatment of the “hydrophobic interactions” between the polar particles and low energy surfaces in aqueous media, as “van der Waals interactions” [91]. Inclusion of acid-base interactions in the classical DLVO approach probably implies both “hydrophobic attractive” and “hydrophilic repulsive” forces. As a result, the interaction force at the primary energy minimum (< several nanometers) can be calculated by the short-range interactions, such as hydrophobic, dipolar, or ionic interactions.

The adhesion of WT was prevented by an addition of 0.02%(v/v) Triton X-100 or 50% ethanol (Fig. 4-11). In these experiments, bacterial cells were strongly aggregated and eventually accumulated in pores of the polyurethane carrier. When the WT cells are tethered to the substratum through the tip of the anchor, the short-range interactions are considered to be functioning between the tip and substratum. Hydrophobic and dipolar interactions are supposed to be prevented by the addition of detergents and polar solvents such as ethanol, respectively. Consequently, it was suggested that WT cells were adhered to the substratum by the long appendages through some kinds of short-range interactions.

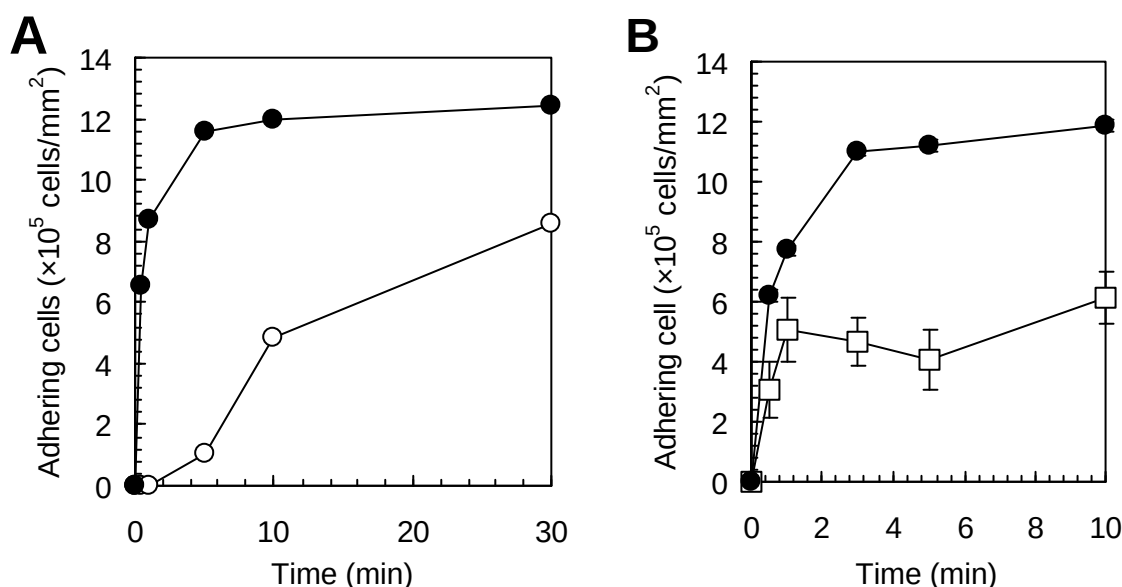


Fig. 4-11 Specific numbers of adhering cells during adhesion tests of WT in the presence of 50% ethanol (**A** - open circle) and 0.02%(v/v) triton X-100 (**B** – open square), compared with the adhesion of WT cells without these reagents (solid circle).

4-3-3. Appendage production dynamics

In order to reveal dependency of appendage formation and adhesiveness on the presence of carriers, Tol 5 cells were grown to stationary phase with toluene as the sole carbon source without the polyurethane carriers. At the stationary phase, cell suspension of WT consisted of individually separated cells (WT-sus) and aggregated cells (WT-agg) (Fig. 4-12). The WT-agg and WT-sus could be separated by leaving the cell suspension on the static condition for 30 min. Then these WT-agg and WT-sus cells were subjected to the adhesion and detachment tests. This detachment test was performed using the polyurethane carrier to which the cells partially adhere during 30 s of the adhesion test, as described above. As shown in Table 4-1, WT-agg cells instantaneously adhered to the polyurethane carrier as well as WT cells that were grown in the presence of the carrier (WT-ad). Adhesiveness of WT-agg is no less strong than that of WT-ad. Furthermore, well like WT-ad, WT-agg cells that had adhered to the carrier once hardly detached from the carrier in deionized water. This suggests that WT-agg cells can irreversibly adhere to the polyurethane carrier in an instant.

In the case of WT-sus, only 18% of the total cells adhered to the carrier within 30 s of incubation. This implies that adhesiveness of WT-sus is lower than 35% of that of WT-ad. After 30min of incubation with the carrier, however, adhered cell fraction reached 79% of the total cells. This value is considerably high compared with that of T1. Synthesis of the

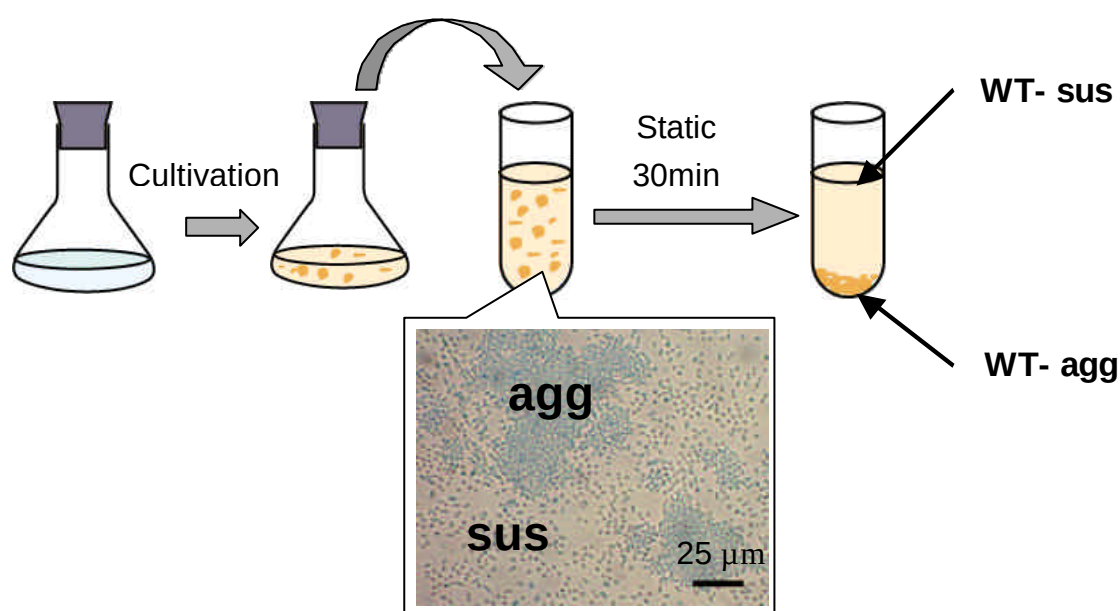


Fig. 4-12 Experimental scheme of the separation of the aggregated (**WT-agg**) and suspended WT cells (**WT-sus**). WT cells were cultivated in the absence of the polyurethane carrier. The cell suspension containing some cell aggregates was standing for 30 min. The sediments were divided as WT-agg.

Table 4-1 Adhesion and detachment tests of WT-agg and WT-sus

Strain	Cell adhesion ($\times 10^5$ cells/mm ²) [†]		Detachment cell fraction from the carrier (%) [‡]
	30 s	30 min	
WT-ad*	6.9 $\pm$ 1.3	12.6 $\pm$ 0.1	1.4 $\pm$ 1.1
T1*	1.0 $\pm$ 0.1	1.9 $\pm$ 0.5	43.6 $\pm$ 8.7
WT-agg	5.8 $\pm$ 0.7	12.7 $\pm$ 0.0	1.7 $\pm$ 0.0
WT-sus	2.4 $\pm$ 0.4	10.3 $\pm$ 0.9	23.1 $\pm$ 5.4

* The cells were cultured at the stationary phase in the presence of carrier.

[†] Specific numbers of adhering cells during adhesion tests

[‡] Detached cell fraction from the carrier during 30 min of incubation of the carrier with the adhered cells in deionized water (%)

appendages may have been involved in the strengthening of adhesiveness during incubation. Meanwhile, during detachment test, a proportion of the adhered cells of WT-sus detached from the polyurethane carrier, which is considered to represent the transition from the initial reversible attachment to secondary irreversible adhesion like T1 cells.

To reveal cell surface appendages, WT-sus and WT-agg cells were observed under TEM with negatively stained by phosphotungstic acid (Fig. 4-13). Almost all of WT-sus cells were settled onto membrane with peritrichate pili-like appendages without aggregation (Fig. 4-13 B), but some WT-sus cells were connected by an anchor-like appendage (Fig. 4-13 A). WT-agg cells could not obtain clear images of cell appendages (Fig. 4-13 C) as similar as T6 cells which are negatively stained by phosphotungstic acid (Fig. 3-14 A). WT-agg cells revealed some anchors to connect each cell body, however, pili-like appendages were not revealed. After dispersion of WT-agg cells by adequate sonication treatment, the cells revealed the peritrichate appendages which resemble pili-like appendages in their morphology (Fig. 4-13 D).

To clarify the relationship between production of the cell appendages and the adhesiveness of WT-sus and WT-agg cells, the cells that adhered to the polyurethane carrier through adhesion test for 30 min observed by FE-SEM (Fig. 4-14). While WT-agg cells as well as WT-ad cells have both the anchor and pili-like appendages on their cell surface, they were hardly seen at all on WT-sus cells. This is inadequate to the images of WT-sus cells with pili-like appendages under TEM. It is considered that pili-like appendages are not important for bacterial adhesion to the polyurethane carrier or only WT-sus cells with pili-like appendages were picked up through the sample preparation for TEM observation.

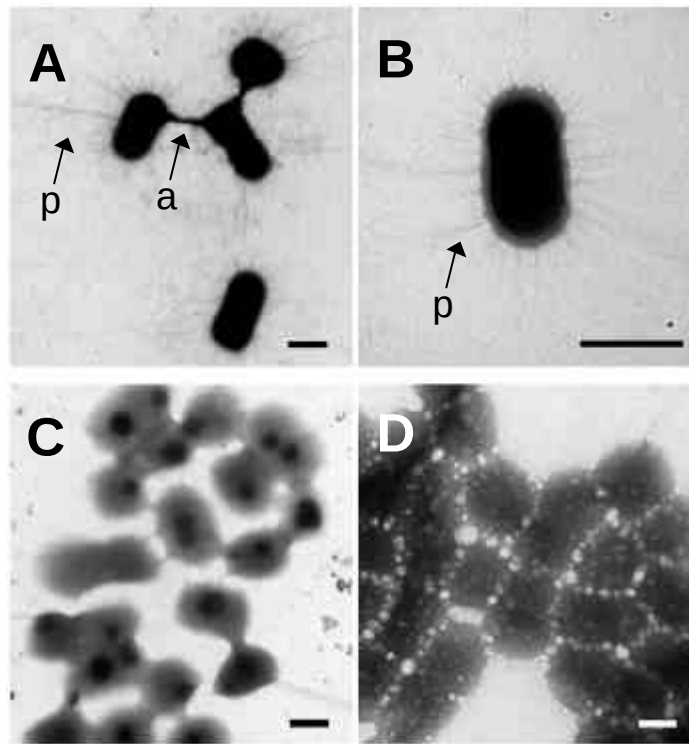


Fig. 4-13 Representative images of cell surface morphology of WT-sus and WT-agg cells under TEM with negatively stained by phosphotungstic acid. WT-sus cells have pili-like (**p**) and anchor-like (**a**) appendages (**A**, **B**). WT-agg cells cannot reveal clear images of the appendages (**C**). After dispersion by adequate sonication treatment, WT-agg cells revealed the peritrichate appendages (**D**). Bars = 500 nm.

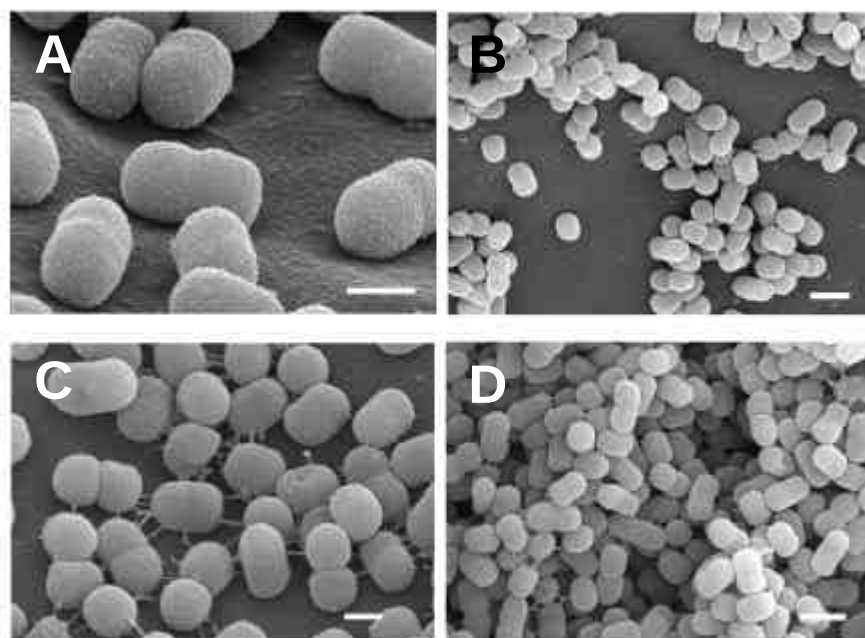


Fig. 4-14 Representative images of bacterial adhesion to the polyurethane carrier of WT-sus and WT-agg cells. WT-sus cells were incubated with polyurethane carrier for 30 min (**A**, **B**). WT-agg cells were incubated with polyurethane carrier for 30 min (**C**, **D**). The close-up images show cell surface morphology (**A**, **C**). Bars = 500 nm (**A**, **C**) or 1 μ m (**B**, **D**), respectively.

These results about the expression of the appendages are consistent with the adhesiveness of WT-ad, WT-agg, and WT-sus. This suggests that the production of the appendages (especially anchor) of a Tol 5 cell is stimulated by the presence of the polyurethane carrier.

Fig. 4-15 showed the dependency of adhesiveness of WT-agg and WT-sus to the polyurethane carrier on the ionic strength. Adhesiveness of WT-sus cells decreased from 10.3×10^5 to 2.1×10^5 cells/mm² with decrease in the ionic strength in the range of 0 mM to 340 mM, like T1 cells. This suggests that electrostatic interaction at low ionic strengths crucially affects the adhesion process on WT-sus cells (see Fig. 4-4). In contrast, WT-agg cells retained adhesiveness in spite of decrease in the ionic strength like WT-ad. This suggests that the WT-agg cells achieve the firm adhesion unaffectedly by the electrostatic repulsive interaction (Fig. 4-14). In other words, while WT-agg follows the DLVO theory, WT-sus does not. These results indicate that the existence of the cell surface appendages strongly affects adhesion dynamics. It is considered that while anchor-like appendages on WT-agg cells mediate the one-step adhesion mechanism proposed in previous section (Fig. 4-11), WT-sus cells adhered to the carrier on conventional two-step adhesion mechanism (Fig. 4-3).

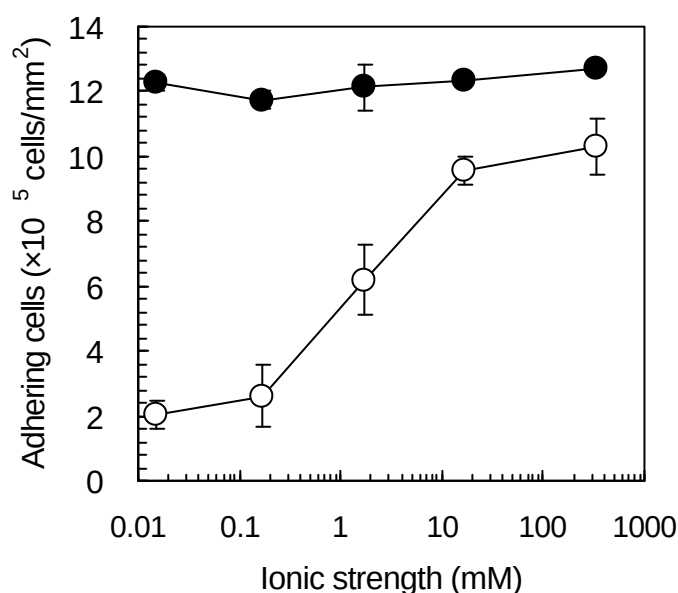


Fig. 4-15 Dependency of adhesiveness of bacterial cells to the polyurethane carrier as a function of ionic strength. WT-agg cells (solid circle) and WT-sus cells (open circle) were incubated with the polyurethane carrier for 30 min at 28°C, 115 rpm, followed by determination of the cell adhered to the carrier (n=5).

4-3-4. Transition of the appendage production

The ratio of WT-sus cells versus WT-agg cells were analyzed at the stationary phase in the 100 ml Erlenmeyer flask without polyurethane carrier. Some Tol 5 cells were adhered to glassware of the flask surface (WT-ad) and cell suspension (WT-sus) including some numbers of cell aggregates (WT-agg) was shown in the liquid phase (Fig. 4-12). Fig. 4-16 shows the cell amounts of WT-sus, WT-agg, and WT-ad cells correlated with the increase of total amounts of WT cells. WT-ad cells were most increased with excessive cells adhering to the glass surface at the medium-air interface. Aggregation ratio in the medium is calculated by a following equation.

$$\text{Aggregation ratio(\%)} = \frac{\text{WT agg}}{\text{WT agg} + \text{WT sus}} \times 100$$

As a result in Fig.4-16, aggregation ratio is 15.7% at 1.1×10^{10} cells/flask and 17.7% at 1.5×10^{10} cells/flask, respectively. These results suggest that the ratio of WT-sus cells versus WT-agg cells were a constant value of approximately 15%, hence it is considered that collision frequency between two cells in the solution might be important to produce cell aggregates and cell appendages. Although aggregation ratio is approximately constant in most cases, large amount of cell aggregate was often produced by some kinds of stimulation to produce cell aggregates and appendages. Consequently, the trigger to produce cell aggregates and appendages is not known in this analysis.

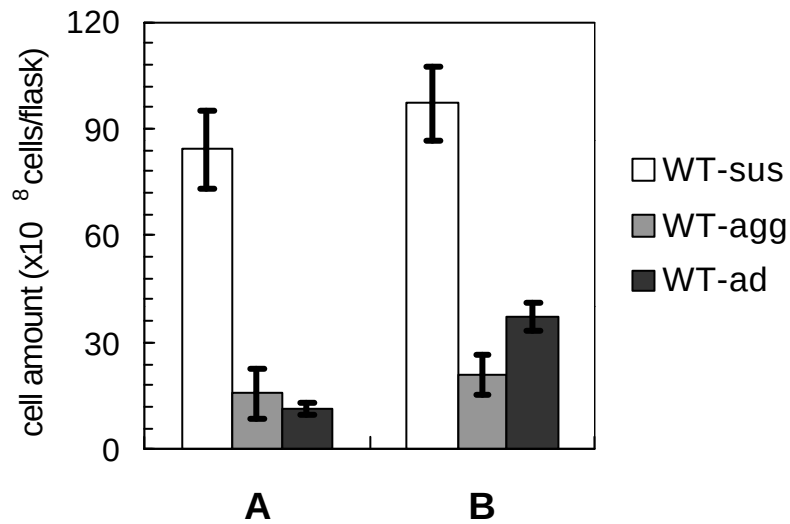


Fig. 4-16 Cell amount of WT-sus, WT-agg, and WT-ad cells in the 100 ml Erlenmeyer flask at the stationary phase without polyurethane carrier. (A) total cell amounts in the flask are 1.1×10^{10} cells. (B) total cell amounts in the flask are 1.5×10^{10} cells. The values are means $\pm$ standard error (n = 3).

To clarify whether the transition from WT-sus cells to WT-agg cells is caused by the irreversible transformation of the genetic character, WT-sus cells and WT-agg cells were individually used as an inoculation source for their subcultures without polyurethane carrier. Aggregation ratio of the subcultures was 10.5% of WT-sus cells and 16.1% of WT-agg cells, respectively. This result suggests that the transition from WT-sus cells to WT-agg cells is not caused by the irreversible transformation of the genetic character, but the transition is caused by the change of environmental factor.

Fig. 4-17 showed the change of adhesiveness of WT-sus cells to the polyurethane carrier with time in condition of deionized water ($I = 0$ mM). Adhesiveness of WT-sus cells increased to 21% (2.7×10^5 cells/mm²) with time. This suggests that electrostatic interaction at low ionic strengths prevents the adhesion of WT-sus cells (see Fig. 4-4). However, WT-sus cells with the addition of 5 μ l of toluene to the deionized water as a carbon source could adhere to the carrier quite larger than without addition of toluene (Fig. 4-17). This suggests that WT-sus cells could achieve the adhesion to the substratum with the addition of toluene in spite of the energy barrier at the low ionic strength. Although the WT-sus cells were difficult to grow in this condition because of adding no nitrogen source, WT-sus cells might produce the appendages to penetrate the energy barrier.

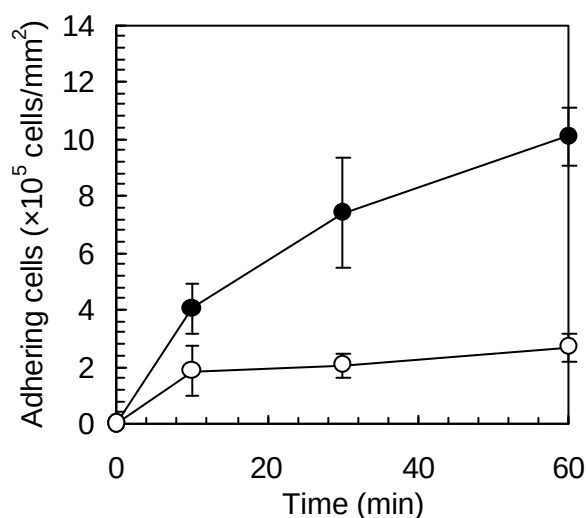


Fig. 4-17 Specific numbers of adhering cells during adhesion tests of WT-sus cells in deionized water ($I = 0$ mM) with toluene as a carbon source (solid circle), compared with the adhesion of WT-sus cells without toluene (open circle). (n=3).

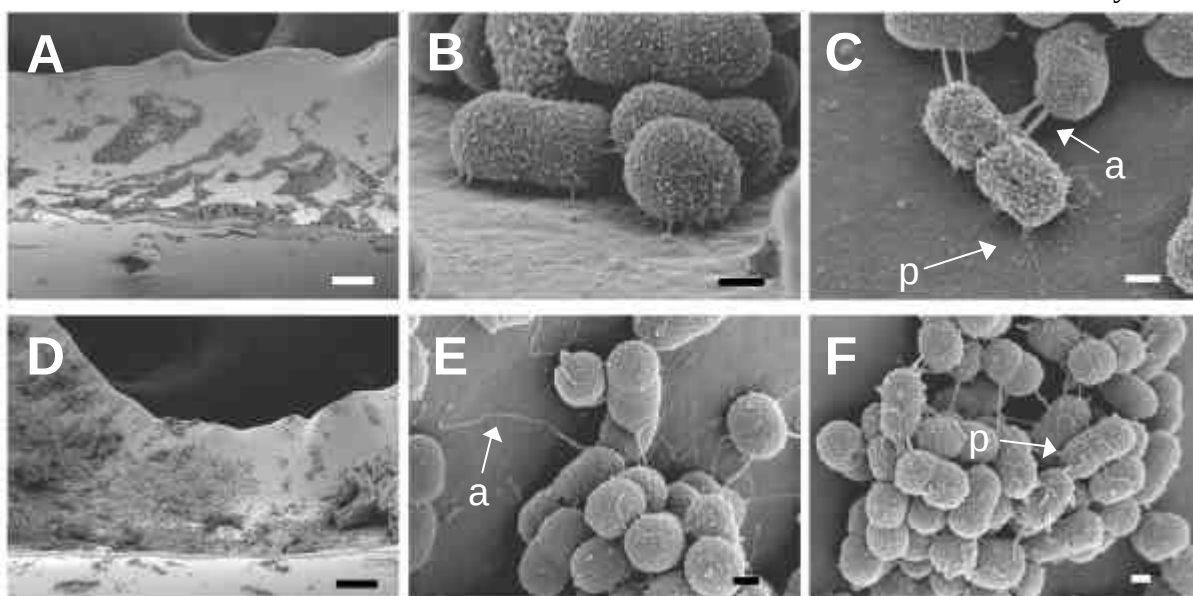


Fig. 4-18 Representative images of bacterial adhesion to the polyurethane carrier of WT-sus cells with/without toluene supplement. WT-sus cells were incubated with polyurethane carrier for 30 min in deionized water ($I = 0$ mM) with toluene as a carbon source (**D - F**), compared with the adhesion of WT-sus cells without toluene (**A - C**). Anchor-like (**a**) and pili-like (**p**) appendages were produced with time. Bars = 10 μ m (**A, D**) or 300 nm (**B, C, E, F**), respectively.

To clarify the appendages production with the addition of toluene and polyurethane carrier in condition of deionized water ($I = 0$ mM), the polyurethane carrier at 30 min of adhesion test was observed by FE-SEM (Fig. 4-18). Without toluene, WT-sus cells showed single layer adhesion to the carrier by using pili-like or short appendage to penetrate the energy barrier formed at the low ionic strength (Fig. 4-18 A-C). With the addition of toluene, WT-sus cells produced many cell surface appendages to adhere to the polyurethane carrier in the form of multi-layer cell clusters (Fig. 4-18 D-F). These results showed that the production of cell surface appendages was induced by the existence of polyurethane carrier as a substratum and toluene as a carbon source. It suggests that WT-sus cells produce the appendages through the recognition of the substratum in some way.

4-3-5. Distribution of appendages in the multi-layer cell cluster

A. genospecies Tol 5 cells adhered to the polyurethane carrier in the form of multi-layer cell clusters. The distribution of appendage production in the cell clusters was unknown, because FE-SEM observation revealed only surface structure of cell clusters. The appendage formation in the cell clusters is important to elucidate the appendage production dynamics of Tol 5 cells. Fig. 4-19 showed the appendages production in the cell clusters after

separation of aggregated cells through some treatments. By trypsin treatment, proteinous appendages were digested, resulting in some debris on the cell surface (Fig. 4-19 D). After cell removal through trypsin treatment, internal cells were revealed on the surface of the polyurethane carrier. The cells possess large number of pili-like appendages and form tight aggregation (Fig. 4-19 C). After the large cell aggregates were separated through strong shear stress by vigorous shaking, Tol 5 cells adhered to the polyurethane carrier in the form of single layer (Fig. 4-19 E). The cells revealed a flexible cell network mediated by anchors on the substratum (Fig. 4-19 F). These images suggest that the anchor is produced at the first step to form multi-layer cell cluster on the substratum, and the pili-like appendage is produced at the subsequent step to retain large cell aggregation on the substratum against hydrodynamic shear stress. Fig. 4-19 (A, B) also showed anchors on the basis of the cell cluster and most pili-like appendages at the upper part of the cell cluster.

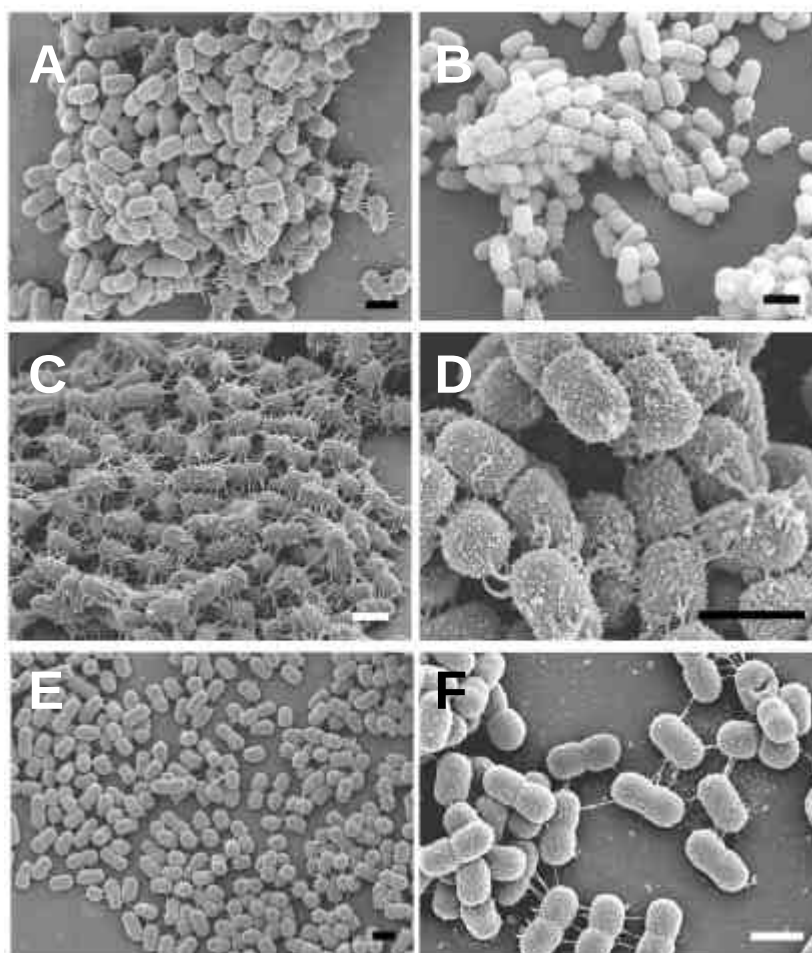


Fig. 4-19 Representative images of adhered cells to the polyurethane carrier of WT-ad cells after separation through some treatments. The surface of the polyurethane carrier after cell removal through vortex (A, B), trypsin treatment (C, D), or strong shear stress (E, F) was observed by FE-SEM. Bars = 1 µm.

4-3-6. Appendage production on the various substrata

In order to investigate the effect of the substratum, WT cells were grown with toluene as the sole carbon source for 16 h in the presence of various substrata such as glass cover slip, epoxy resin, and glass cover slip treated with octadecyltriethoxysilane (hydrophobic glass, see 3-2-3). WT cells adhered well to all of the tested substrata. In particular, WT cells could adhere to the more hydrophobic substrata than hydrophilic substrata (Data not shown). Fig. 4-20 showed that WT cells adhered to the various substrata through some appendages by using FE-SEM. These images showed the different morphology of cell surface appendages of WT cells. On the epoxy resin, many appendages were overexpressed on the surface of WT cells (Fig. 4-20 C, F). Although it is difficult to identify whether these fibrils are the anchor or pili-like appendage, most of these appendages protruding to the substratum or cell body seem responsible for the firm adhesion to epoxy resin. On the hydrophobic glass, the many WT cells adhered to the substratum in a single-layer. Fewer appendages were observed in the WT cells adhered on the hydrophobic glass than those on the untreated glass (Fig. 4-20 A, B). The many pili-like appendages, which directly interact with the surface, can be seen on the untreated glass. The anchor-like appendage tethering the cell body to glass surfaces are also seen (Fig. 4-20, D, E). Moreover, many anchor-like appendages were shown near the large aggregate to connect cell body in the image on the hydrophobic glass (data not shown).

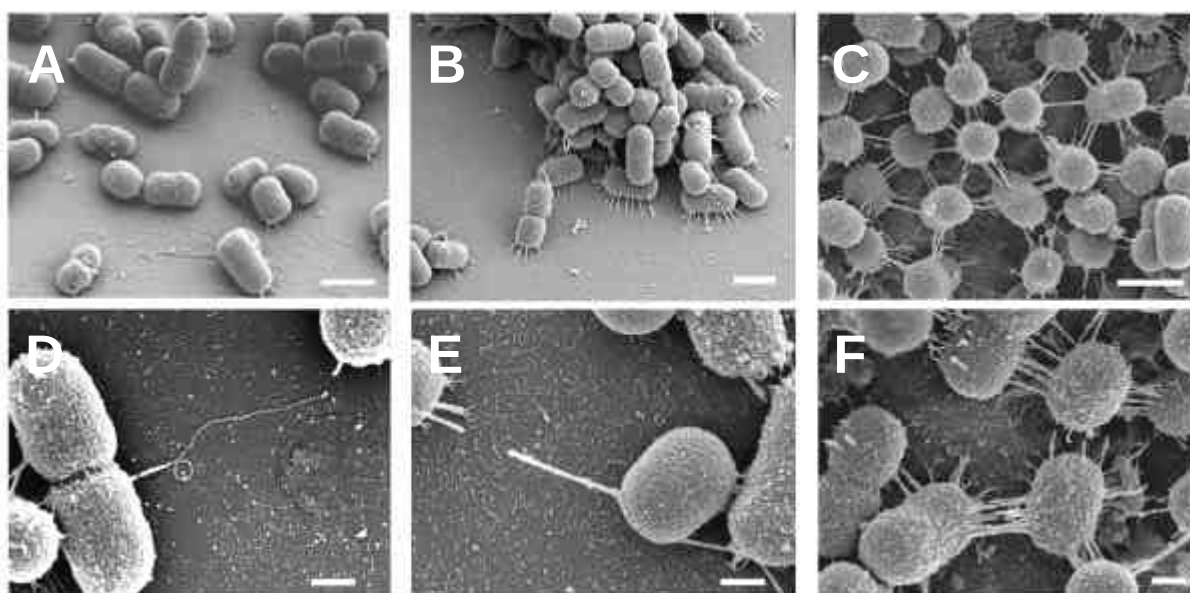


Fig. 4-20 Representative FE-SEM images of bacterial adhesion of WT cells to the hydrophobic glass (**A**, **D**), untreated glass (**B**, **E**), and epoxy resin (**C**, **F**). The close-up images show cell surface morphology (**D** – **F**). Bars = 1 µm (**A** – **C**) or 300 nm (**D** – **F**), respectively.

It is likely that pili-like appendages were induced to adhere the cell body firmly to the substratum through large aggregation formation. Some researchers demonstrated that on attachment-dependent promoter induces cell appendages [55, 84]. There is a possibility that the production of the pili-like appendages of Tol 5 cells are regulated by this type of promoter to adhere firmly. In addition, some gram-negative bacteria possess a quorum-sensing system, in which the bacterial cells recognize the cell density through auto-inducer concentration [63, 75, 76]. It can be considered that the pili-like appendages are expressed through the quorum-sensing system to make tenacious adhesion. It has not been elucidated when the anchor-like appendages are produced and what stimulation effects production of the appendages. Eventually, appendage production dynamics of *A. genospecies* Tol 5 include complicate process. For its elucidation, comprehensive frames such as morphological analysis, characterization, proteomics and genomics are required.

4-4. Summary

The mechanism of bacterial adhesion is important for controlling biofilm formation and microbial colonization – processes that are detrimental to human health and various industrial sectors, but beneficial to environmental biotechnology. *Acinetobacter genospecies* Tol 5 possess the anchor-like appendage that extends straight from the cells to the substratum without branching and its end interacts with the substratum. The bacterial cells use the anchor to tether themselves to the substratum at distances of several hundreds of nanometers. The WT cells attained irreversible adhesion within 30 s and adhered to the substratum independent of electrostatic repulsive force. This suggests that planktonic cells can attain irreversible adhesion in one step through the anchor. This finding changes the general concept of bacterial adhesion, which previously has been considered to be initiated by cells located within tens of nanometers of a substratum.

Additionally, the Tol 5 cells showed the appendages produced by the existence of the substrata. The suspended cells of Tol 5 were harvested in the culture without carrier and did not possess the long appendages. This type of cells (WT-sus) adhered to the substratum by the conventional two-step adhesion process such like appendages-defective T1 cell. This also suggests that the appendages of Tol 5 cells are important for one-step adhesion process.

Chapter V

Conclusions

5-1 General conclusions

Acinetobacter genospecies Tol 5 isolated from the TBAB which had been used for toluene elimination showed high toluene degradation ability, and had high adhesion ability to polyurethane.

Chapter II, “Characterization of *A. genospecies* Tol 5”, describes the general feature of *A. genospecies* Tol 5. The bacterium demonstrated the high efficiency in toluene removal. The bacterium tended to adhere to hydrophobic materials showing water-repellent. A TBAB inoculated with *A. genospecies* Tol 5 showed a sufficiently high ability in toluene elimination within 24 h after starting the operation without preliminary operation for immobilization. This suggested that this strain was effective to shorten the adaptation period, which had been considerably long on the TBAB inoculated with original activated sludge.

Chapter III, “Bacterial adhesion through cell surface appendages”, describes the specific adhesion of *A. genospecies* Tol 5 for strong adhesion ability. *A. genospecies* Tol 5 shows extreme adhesive properties as compared with other adhesive bacteria such as *Pseudomonas aeruginosa* PAO1. A unique anchor-like appendage as well as pili-like appendage has been discovered on Tol 5 cells using transmission electron microscopy (TEM) and field-emission scanning electron microscopy (FE-SEM). The anchor extends straight from the cells to the substratum without branching and its end interacts with the substratum. The bacterial cells use it to tether themselves to the substratum like a balloon on a string at distances of several hundreds of nanometers. This striking feature of the anchor was confirmed to be intact by observation under a variable-pressure FE-SEM, which dispenses with the sample preparation needed for conventional SEM observation. A less-adhesive mutant, T1, has lost these appendages. Protein was found to be an important component of the anchor by trypsin treatment.

Chapter IV, “Adhesion dynamics through anchor-type appendage”, describes the novel adhesion mechanism through the anchor of the *A. genospecies* Tol 5 cell. The Tol 5 cells attained irreversible adhesion within 30 s and adhered to the substratum independent of electrostatic repulsive force. This suggests that planktonic cells can attain irreversible adhesion in one step through the anchor. This finding changes the general concept of bacterial adhesion, which previously has been considered to be initiated by cells located within tens of

nanometers of a substratum. The suspended cells of Tol 5 were harvested in the culture without carrier and did not possess the long appendages. This type of cells (WT-sus) and suspended T1 cells adhered to the substratum by the conventional two-step adhesion process. This also suggests that the appendages of Tol 5 cells are important for one-step adhesion process.

5-2 Prospective points for further study

As a result in Fig. 3-10, unfortunately, the insertion by transposon mutagenesis had occurred not at a single site but at multiple sites, including a homologous recombination site. The DNA sequencing of randomly inserted site will make clear the gene of anchor and pili-like appendages of Tol 5 cells. By a homology analysis, it will become apparent whether the anchor and pili-like appendage are novel appendages or not. If the each genes encoding anchor or pili-like appendage are obtained, the production dynamics of the appendages for bacterial adhesion will enable to be analyzed by Northern blot analysis.

As observations of Fig. 3-16 and Fig. 3-17, the specific proteins were revealed by biotinylation and SDS-PAGE or two-dimensional gel electrophoresis. Up to now, many reports have showed the method of separation and purification of pili and flagella. After purification of the proteins of the anchor and pili-like appendages, SDS-PAGE and two-dimensional gel electrophoresis of them will show more clear band, followed by carried out protein sequence. The pili and flagella have complex assemblies of its component protein. Hence the component analysis of anchor is important to analyze the interaction energy between the tip protein of anchor and the substratum. Contact mode atomic force microscopy enables to show the interaction energy directly between a tip of the cantilever and a specimen. Furthermore the mapping of hydrophobicity in a bacterial cell can be analyzed by AFM. This might show the localization of hydrophobicity in the Tol 5 cell and its appendages.

The appendages production dynamics is most crucial problem to investigate in more detail, because of the heterogeneity of the appendages production in Tol 5 cells. In this study, Fig. 3-6, 4-18 to 20 showed various types of Tol 5 cells that expressed the appendages. The many possibility are suspected by following aspects of Tol 5 cells:

- Adhesiveness of WT-sus cells is increased at the addition of toluene at the condition of 0 mM of ionic strength (Fig. 4-17). In this transition, WT-sus cells produce the appendage to adhere to the substratum (Fig. 4-18). Which stimulation is a trigger to produce cell appendages?
- Adhesiveness of WT-sus cells is increased by the change from the culture medium at the stationary phase (24% of adhesion ratio) to fresh BS medium (72% of adhesion ratio). This suggested that the culture medium including secondary metabolism products prevent the bacterial adhesion of WT-sus cells. How the culture medium prevent the bacterial adhesion of WT-sus cells?
- Adhesiveness of WT-sus cells is increased by the centrifugation (from 24% to 43% of adhesion ratio). Which change is occurred to the WT-sus cells through the centrifugation treatment?

Tol 5 cells showed the various aspects of appendages production with the change of growth phase, the feature of substratum, and the kind of carbon source. The anchor and pili-like appendages might be developed by some stimulation from the change of culture condition. The investigation of this stimulation will obtain further information of bacterial adhesion mechanism of Tol 5 cells.

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List of publications

- (1) **Hori, K., S. Yamashita, S. Ishii, M. Kitagawa, Y. Tanji, and H. Unno.** (2001) Isolation, characterization and application to off-gas treatment of toluene-degrading bacteria., J. Chem. Eng. Jpn. **34**:1120-1126.
- (2) **Ishii, S., J. Koki, H. Unno, and K. Hori.** Current electron microscopic techniques reveal unique cell appendages on a strongly adhesive bacterium, *Acinetobacter genospecies* Tol 5., Appl. Environ. Microb. (submitted)

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