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Drastic change in cell surface hydrophobicity of a new bacterial strain, *Pseudomonas* sp. TIS1-127, induced by growth temperature and its effects on the toluene-conversion rate

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In a previous study, we reported the effectiveness of a bacterial strain showing monolayer adsorption to oil surfaces on microbial conversion at oil–water interfaces. In the present study, we screened wild type strains from our toluene-degrading bacterial library that showed similar properties and succeeded in obtaining five wild type strains that adsorb to oil surfaces as a cell monolayer. We investigated the effects of cultivation conditions on cell surface hydrophobicity of these five strains. The effects of substrate hydrophobicity and the porous carrier were not significant. By contrast, growth temperature greatly affected the cell surface hydrophobicity of all five strains, especially strain TIS1-127, which was phylogenetically identified as *Pseudomonas* sp. which is closely related to *P. mosselii, P. monteilii*, and *P. plecoglossicida*. *Pseudomonas* sp. TIS1-127 cells grown at 37 °C were determined by the kinetic microbial-adhesion-to-hydrocarbon (MATH) test to be fully hydrophilic (lower than 10% of MATH value) while the cells grown at 28 °C were highly hydrophobic (over 90% of MATH value). We investigated the effects of growth temperature on toluene conversion by TIS1-127 resting cells in single-phase batch cultivation and in two-liquid-phase partitioning reactors containing an emulsion consisting of 20% silicone oil and 80% cell suspension. In both cases, the cells grown at 28 °C showed much higher conversion ability than those grown at 37 °C. Toluene conversion followed Michaelis–Menten kinetics and the K_m values for the cells grown at 28 °C were lower than 1/10 those for the cells grown at 37 °C.

[**Key words:** *Pseudomonas*; Two-liquid-phase partitioning bioreactor (TPPB); Emulsion; Toluene conversion; Cell surface hydrophobicity; Growth temperature]

The surface hydrophobicity of microbial cells is an important property that directly affects the efficiency of various bioprocesses, such as bioremediation, waste treatment, and green biotechnologies, using whole microbial cells. The microbial-adhesion-to-hydrocarbon (MATH) test allows for direct evaluation of the behavior of microbial cells in a two-liquid-phase system and the interaction between the cells and an organic phase, including the affinity of the cells for the organic surface, although the outcome of this test is affected not only by hydrophobic interactions but also by van der Waals and electrostatic interactions (1, 2). Therefore, this method provides the best index when considering and designing systems in which microbial conversion at the interface between aqueous and organic phases is expected. These systems have received increasing attention for use in bioremediation and/or treatment of oil-contaminated aqueous fields, as well as for microbial conversion in two-liquid-phase partitioning reactors (TPPBs) (2–6).

TPPBs consist of an aqueous phase containing microorganism cells and a water-immiscible, non-bioavailable organic phase. In TPPBs, the organic phase acts as a substrate reservoir and keeps microorganisms in the aqueous phase separated from high concentrations of toxic and hydrophobic substrates and/or products in the organic phase. TPPBs have shown excellent performance and high stability in a variety of applications, such as in the degradation of VOCs in off-gas as well as in green production of aromatic chemicals including catechol (7), phenol, 2-naphthol (8), *o*-cresol (9), and (*R*)-phenylacetylcarbinol (10), and non-aromatic chemicals including nisin (11).

The toluene-degrading bacterium, *Acinetobacter* sp. Tol 5, which was isolated from a biofilter system for off-gas treatment that contained toluene, has a hydrophobic cell surface and high adhesiveness to solid surfaces (12). Tol 5 cells have filamentous appendages that are responsible for the high adhesiveness and self-agglutinating property (13, 14). Bald cells of a mutant strain (T1) of this bacterium lack these filamentous appendages and show decreased adhesiveness, but retain

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the hydrophobic cell surface (15). Owing to this lack of cell appendages responsible for the self-agglutinating property, T1 cells adhere without cell clumping in a monolayer to hydrocarbon surfaces dispersed in an aqueous matrix, whereas wild type cells with appendages aggregate on hydrocarbon droplets. The adhesion of T1 cells to the hydrocarbon surfaces is reversible and can be described by the Langmuir adsorption isotherm (1). Langmuir adsorption has potential applications in the development of green technologies employing TPPBs and in bioremediation because bacterial cells can effectively access substrates dissolved in the organic solvent phase, and because the effect of toxic substrates dissolved in the organic solvent phase on the adhering cells can be minimized by substitution with fresh or recovered cells suspended in the aqueous phase. In a previous study, by employing the mutant strain T1 of *Acinetobacter* sp. Tol 5 in TPPBs, we achieved a toluene conversion rate of 3.1 g/(*l*·h), which was 10-fold faster than any experimental data previously demonstrated (16).

In this study, we obtained a new toluene-degrading wild type strain, which shows monolayer adsorption to hydrocarbon surfaces. Here, we show a drastic change in the cell surface hydrophobicity of this strain induced by growth temperature as well as the effects of the growth temperature on the capability for toluene conversion of this strain.

MATERIALS AND METHODS

Bacterial strain and culture conditions The strain TIS1-127, which was isolated as a toluene-degrading bacterium from soil and stocked in a toluene-degrading bacterial library (17), was conventionally cultivated in 100 ml of basal salt (BS) medium (13) supplemented with 0.5 ml/*l* toluene in a 500-ml Erlenmeyer flask with a butyl rubber cap at 28 °C, 115 rpm. To prepare resting cells for toluene conversion experiments, cells were grown in the same way but the flask was incubated at 28 °C or 37 °C. At the stationary growth phase, 50 ml of another toluene was added to the culture in order to reinduce enzymes for toluene degradation 2.5 h before the toluene conversion experiments (16).

To investigate the effects of cultivation conditions on cell hydrophobicity, lactate was used as a hydrophilic substrate to substitute for toluene, a hydrophobic substrate. A sponge carrier made of polyurethane (15) was used as a porous carrier for cell adhesion.

Phylogenetic analysis Chromosomal DNA was extracted from strain TIS1-127 cells grown in BS medium supplemented with toluene using the Isoplant II DNA extraction kit (Nippon Gene, Tokyo, Japan) according to manufacturer's protocol. The sequence of 16S rDNA was amplified by PCR using the extracted chromosomal DNA as a template and a primer set of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) (18). The sequence of the amplified 16S rDNA fragment was determined by the primer walking method. The obtained 16S rDNA sequence was subjected to phylogenetic analysis by CLUSTAL W (19) and MEGA3 (20) using the neighbor-joining method (21) against the sequences of type bacterial strains selected from the DDBJ/EMBL/GenBank on the basis of homology analysis by BLAST (22).

MATH test Bacterial cells were harvested at the stationary growth phase by centrifugation, washed with sterile water, and resuspended to an optical density at 660 nm (OD_{660}) of ~0.5 in fresh ultrapure water produced by reverse osmosis. Aliquots (1 ml) of the cell suspension were transferred to test tubes (100 mm × 10.5 mm internal diameter), and 200 μl of hexadecane or a silicone oil (KF96L-1CS; Shin-Etsu Chemical, Tokyo, Japan) was added. After vigorous vortex mixing for 60 s, the OD_{660} of the aqueous layer was measured. The MATH value was calculated from the change in OD_{660} as follows:

$$\mathrm{MATH}(\%) = \frac{\mathrm{OD}_{660-\text{before treatment}} - \mathrm{OD}_{660-\text{after treatment}}}{\mathrm{OD}_{660-\text{before treatment}}} \times 100.$$

For the kinetic MATH test (1), an equal number of test tubes to time points were prepared and subjected to the MATH test described above. Each test tube was vortexed until the time point for measurement, and 800 μl of the aqueous phase was transferred to a cuvette for measurement of OD_{660}. To avoid any reduction of volume, different test tubes were used for each measurement. The MATH values obtained from each time point were plotted against vortex time.

Toluene conversion experiment Bacterial cells were harvested by centrifugation at 28 °C, rinsed twice with BS-N medium, and resuspended in BS-N medium. BS-N, which lacks nitrogen, was used to prevent growth and *de novo* protein synthesis during the toluene conversion tests (16). The cell concentration of the suspension was adjusted according to OD_{660}; an OD_{660} of 1.0 corresponded to 0.45 g dry cell weight per liter.

The toluene conversion tests in single-liquid phase reactors were carried out in 125 ml cylindrical glass vials containing 20 ml of the cell suspension and a stirring chip. The headspace gas was replaced with oxygen by blowing pure oxygen gas for 3 min, followed by immediate sealing with a butyl rubber plug and an aluminum cap. Oxygen inhibition of the conversion activity was not observed. Finally, toluene was injected using a gas-tight syringe, and the vials were placed on a stirrer. The initial applied concentrations of toluene and cells were varied. The vials were incubated at 28 °C for toluene conversion by the resting cells. Periodically, 50 μl of the headspace gas was analyzed by gas chromatography (GC) as described below.

The toluene conversion tests in the TPPBs were carried out in 125 ml cylindrical glass vials containing 16 ml of the cell suspension and 4 ml of silicone oil (same as for the MATH tests). The properties of the silicone oil were as follows: kinematic viscosity at 25 °C, 1.0 mm^2/s; specific gravity at 25 °C, 0.818; and boiling point, 153 °C. Toluene conversion was initiated using the same procedure as described for the single-liquid phase reactor. The oil and aqueous phases were emulsified by vigorous mixing, and the vials were incubated at 28 °C. The vials were collected from the stirrer at selected times. The liquid in the vials was decanted into centrifuge tubes, which were immediately capped and then centrifuged at 10 000 ×*g* for 1 h at 28 °C in order to separate the oil and aqueous phases. The loss of toluene caused by volatilization was negligible during this sampling procedure. The toluene concentration of the oil phase was quantified by GC as described below. The initial conversion rate was determined from the decrease in the toluene concentration of the oil phase during the initial 30 min before the consumption of oxygen and toluene decreased the conversion rate. During this period, conversion rates showed zero order reactions in every condition tested (16).

Experimental data were plotted on graphs of substrate concentrations versus conversion rates (*S*–*V* plot) and simulation curves were drawn assuming Michaelis–Menten kinetics using the Solver function of Microsoft Excel.

Measurement of the organic solvent tolerance Bacterial cells were precultured on toluene at 28 °C or 37 °C for 24 h as described above. After harvesting the cells by centrifugation, they were resuspended in BS medium to an OD_{660} of 1.0. The diluted cell suspension was spotted on LBGMg agar plate (23). Approximately 10^7, 10^6, 10^5, 10^4 and 10^3 cells were contained in the spots. The organic solvent-tolerance level of the cells was determined by measuring colony formation after 24-h incubation at 28 °C on the agar plate overlaid with organic solvents (24, 25). Cyclohexane, toluene, and their mixture (cyclohexane:toluene = 7:3) were used as the solvents.

Analytical methods The toluene concentration in silicone oil was quantified by a GC system (GC-17A, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a ULBON HR-1 capillary column (50 m × 0.25 mm inside diameter; Shinwa Chemical Industries, Kyoto, Japan). Before injection of 2 μl of the oil samples into the GC system, 500 μl of the oil samples containing toluene was mixed with 500 μl of the silicone oil containing 1 μl of hexadecane as an internal standard. Toluene concentrations in the silicone oil were determined from the standard curve of toluene concentrations against ratios between toluene and hexadecane peak areas. Because the toluene concentration of the aqueous phase was lower than the GC detection limit, the toluene concentration in the silicone oil was used to calculate the toluene concentration of the emulsion (i.e., the liquid phase). Headspace gas was directly injected into the GC system to determine its toluene concentration. The aqueous concentration of toluene in BS-N medium at 28 °C was calculated using dimensionless Henry's constant, which was experimentally determined previously (26).

The stable emulsion obtained in the MATH test was observed using a digital microscope with a ultra long working distance lens (Keyence VHX-200 microscopes with a VH-Z450 lens) in order to visualize cells adhering to spherical hexadecane droplets with diameters of several tens to hundreds of micrometers.

Nucleotide sequence accession number The 16S rDNA sequence datum has been submitted to the DDBJ/EMBL/GenBank under accession no. AB456678.

RESULTS

Screening of wild type strains showing monolayer adsorption to oil surfaces Owing to the discovery of the effectiveness of a bacterial strain showing monolayer adsorption to oil surfaces on microbial conversion at oil–water interfaces (16), here we screened wild type strains from our toluene-degrading bacterial library that showed similar properties. From the bacterial library consisting of 97 strains showing different repetitive extragenic palindromic patterns (17), 5 strains including strain TIS1-127 were selected as showing high MATH values but no self-agglutinating property. These strains stabilized emulsion formed by the MATH tests (Fig. 1A). Although many strains showing high MATH values self-agglutinated like strain TIS2-35 (Fig. 1B), cells of these 5 strains did not self-agglutinate and were suspended in the culture medium as individually dispersed cells (Fig. 1C). Monolayer adsorption of these cells to hexadecane droplet surfaces was confirmed by microscopy (Fig. 1D). Thus, we succeeded in obtaining wild type strains that adsorb to oil surfaces in a cell monolayer similarly to the mutant strain T1 of *Acinetobacter* sp. Tol 5 (1).

Effects of cultivation condition on cell surface hydrophobicity We investigated the effects of cultivation condition on the cell surface hydrophobicity of the selected 5 strains. The effects of sub-