Characterization of Floating Activity of Indigenous Diesel-Assimilating Bacterial Isolates

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Six diesel-degrading bacterial strains were isolated from oil-polluted sites located in central Taiwan. The floating activity of the isolates in an oil-supplemented liquid medium was monitored. Cell-surface hydrophobicity as well as cell-free and cell-residue emulsification activities were also investigated. Three isolates, identified as Gordonia alkanivorans CC-JG39, Rhodococcus erythropolis CC-BC04, and R. erythropolis CC-BC11, were found to float and grow near the diesel layer on the surface. The other three isolates (namely, Comamonas testosteroni CC-CF3, Acinetobacter sp. CC-CF5, and Sphingomonas yanoikuyae CC-CG22) did not display floating activity, as they distributed uniformly in the liquid medium. Isolated cell walls of the floating strains appeared to settle at a lower sucrose density than the nonfloating strains. The floating strains were also characterized by a higher cell-surface hydrophobicity and a higher cell-residue emulsification activity than the nonfloating strains. In fact, the floating strains were thought to produce extracellular emulsifiers due to their higher supernatant emulsification activity than the nonfloating strains. The floating activity of G. alkanivorans CC-JG39 may be associated with the production of extracellular polymeric substances that formed an “air-bag” structure facilitating cell floating. The floating ability may also correlate with a high cellular hydrophobicity arising from unique cell wall compositions or cell-wall-bound surface active products.

[Key words: diesel biodegradation, floating, emulsification, cellular hydrophobicity]
and the enhanced uptake of oil substrates were proposed.

**MATERIALS AND METHODS**

**Isolation, selection and identification of diesel-degrading bacteria** The strains of bacteria used in the study were isolated from various oil-contaminated sources, including the seashore, landfill soil and oil-polluted soil near gas stations. Samples of 10 ml liquid or 5 g soils from the polluted sites were transferred into 250 ml flasks containing 100 ml of the liquid mineral BH medium (K2HPO4, 1.0 g/l; KH2PO4, 1.0 g/l; NH4NO3, 1.0 g/l; MgSO4·7H2O, 0.2 g/l; CaCl2, 0.2 g/l) supplemented with 1.0% (v/v) diesel oil as the sole carbon source. The liquid cultures of potential diesel-degrading bacteria were incubated at 30°C in an orbital shaker (180 rpm) for 1 week. Strains with optimal diesel-degrading activity were then selected from the aforementioned liquid culture by repeated subcultures and purifications on the nutrient broth (NB) agar medium containing 3 g/l yeast extract, 5 g/l peptone, and 15 g/l agar. The diesel-degrading isolates were identified by 16S rDNA analysis using an ABI 310 automated genetic analyzer (Applied Biosystems, New York, NY, USA).

**Cell growth on diesel-supplemented medium** Single colonies on the NB agar medium cultured for 48 h were transferred into the BH liquid medium containing 0.1% (v/v) diesel in 500 ml shake flasks. The liquid cultures were incubated at 30°C and agitated at 160 rpm. Samples were taken to measure cell density and residual diesel content (by gas chromatography) at designated time intervals.

**Diesel analysis** A liquid sample (50 ml) was extracted twice with 50 ml of dichloromethane (1:1 v/v). The organic phase was passed through anhydrous Na2SO4 to remove residual water and was concentrated to 0.2 ml by using a vacuum evaporator. The resulting product was again dissolved in 2 ml of dichloromethane and concentrated to 1 ml by purging N2 gas. The concentrated sample (1 μl) was injected into a gas chromatography (model 5890II; Hewlett Packard, Washington, D.C., MD, USA) equipped with a flame ionization detector and a capillary column (30 m in length, 0.53 mm inner diameter, D.F. 1.5 μm, type RTX-5; Restek, New York, NY, USA). Peaks from retention time within 10–28 min were used for total diesel determination in all experiments. To control the percentage of diesel recovery, commercial standard diesel oil (1000 mg/l) was added to 50 ml of uninoculated medium and then subjected to the same diesel analysis procedures as used for inoculated samples. About 85% of diesel was recovered in the control trial at the end of the experiments. All the experiments were performed in duplicate or triplicate.

**Floating test** A bacterial culture (0.4 ml of nutrient broth preculture) was inoculated into a 78 ml test tube (220 mm in length and 24 mm in diameter; Pyrex, Los Angeles, CA, USA) containing 40 ml of the liquid mineral BH medium supplemented with 1% (v/v) diesel oil as the sole carbon source. The culture was maintained at 30°C with agitation at 160 rpm for 4 d and was incubated for another 24 h without agitation. Aliquots of 1 ml were then taken from a culture using a sterile syringe at 0, 1, 5 and 10 cm depths from the surface. Spread plate count was carried out and the spatial distribution of cells was estimated by counting the colony-forming units (CFU) of samples collected at different depths. All the experiments were performed in duplicate or triplicate.

**Sucrose density gradient centrifugation** Cell walls of the test strains were isolated according to the procedures suggested by Hirschfield et al. (12). Cell wall fragments or whole cells were washed thoroughly with distilled water and PBS and were prepared as described by Hirschfield et al. (12). The suspensions of cells or cell wall fragments (with an optical density of about 0.7–0.8 at 600 nm) were gently loaded on the top of a discontinuous sucrose gradient of 0%, 5%, 10%, 20%, 30% (v/v) sucrose. The procedures of density gradient centrifugation for whole cells and isolated cell walls were similar to those reported by Glaser et al. (13) and Hirschfield et al. (12), respectively. Samples were then carefully taken from each sucrose concentration layer using a syringe and were subjected to viable cell count or turbidity measurement to determine the distribution of whole cells or cell wall fragments in the sucrose density gradient. All the experiments were performed in duplicate or triplicate.

**Determination of cellular hydrophobicity** The cellular hydrophobicity of tested strains was determined according to the procedures described by Rosenberg et al. (10). In brief, cells collected from the preculture were rinsed twice with sterile deionized water and the rinsed cells were suspended in PUM buffer (K2HPO4·3H2O, 22.2 g/l; KH2PO4, 7.26 g/l; urea, 1.8 g/l; MgSO4·7H2O, 0.2 g/l) to reach a final volume of 1.2 ml and an optical absorbance (at 400 nm) of 9.0±1.0 (i.e., a cell concentration of 1.3±0.3 mg/l). Various volumes (0–0.2 ml) of hydrocarbons tested (kerosene or diesel) were added into the cell suspensions, which were then incubated at 30°C for 10 min. The mixtures were subsequently agitated thoroughly with a vortex mixer (model VM-200; Digisystem Laboratory Instruments, New York, NY, USA) for 120 s. Then, the mixtures were allowed to stand for 15 min to separate the hydrocarbon and aqueous phases. Samples were taken carefully from the aqueous phase and the absorbance at 400 nm (OD400) of the samples was measured using a spectrophotometer (model U-2001; Hitachi, Tokyo). Cellular hydrophobicity is defined as follows:

\[
\text{Hydrophobicity (\%) = } \frac{\text{OD}_{400} \text{ of aqueous phase of culture after mixing with hydrocarbon}}{\text{OD}_{400} \text{ of initial culture prior to hydrocarbon addition}} \times 100\% \tag{1}
\]

**Emulsion test** The six isolated strains were cultivated in the diesel (1% v/v)-containing HB medium until the early-stationary phase (ESP) (cell concentration was about 1.5 g/l). The emulsification activity of supernatants or cell residues collected from ESP cultures was determined by measuring the emulsion index (E24) according to the procedures described by Cooper and Goldenberg (14). The supernatant was obtained by centrifugation (10,000 × g; 8 min) and the resulting cell pellet was suspended in deionized water and subsequently sonicated (60–75 Watt; 5 min) using an ultrasonic processor (Sonics & Materials, New York, NY, USA). The cell residue was rinsed twice and resuspended in deionized water for the emulsification test. Four milliliter of the supernatant (or cell residue suspension) was poured into a test tube containing 6 ml of oil (diesel or kerosene). After vigorous mixing using a vortex mixer, the test tube was allowed to stand for 24 h and the emulsion index (E24) was determined using the following equation:

\[
E_{24} (\%) = \frac{\text{Height of emulsified zone}}{\text{Height of total liquid (sum of oil, emulsified, and aqueous zones)}} \times 100\% \tag{2}
\]

**Surface tension measurement** The surface tension of supernatants collected from early-stationary-phase cultures grown on the NB medium (yeast extract, 3 g/l and peptone, 5 g/l) was detected using a FACE Surface tensiometer (model CBVP-3; Kyoto Interface Science, Tokyo).

**Scanning electron microscopy (SEM)** G alkanivorans CC-JG39 cells were cultivated in the BH medium supplemented with 1% (v/v) diesel. An appropriate amount of culture was taken
on the first and fifth days for SEM. Sample preparation for SEM of G. alkanivorans CC-JG39 essentially followed the procedures suggested by Gilpin (15). The major steps included the cleaning of the specimen, fixation, dehydration, and coating. Micrographs were taken using a SEM machine (model ABT150S) manufactured by Topcon (Tokyo).

**RESULTS**

Physiological and floating characteristics of diesel-degrading isolates  
Table 1 shows the results of strain identification as well as the morphological and phenotypic characteristics of the six indigenous isolates. The comparison of 16S rDNA sequences of the six isolates with those in the GenBank shows that these isolates had 98–100% similarity to standard strains (Table 1). The 16S rDNA sequences of the six isolates have been deposited in the NCBI nucleotide sequence database with the following accession numbers: *Rhodococcus erythropolis* CC-BC04, AY864339; *R. erythropolis* CC-BC11, AY864340; *Comamonas testosteroni* CC-CF3, AY864337; *Acinetobacter* sp. CC-CF5, AY864336; *Sphingomonas yanoikuyae* CC-CG22, AY862983; and *Gordonia alkanivorans* CC-JG39, AY864338. All of the six isolates assimilated diesel (Fig. 1) and were also able to utilize other aromatic compounds (Table 1). Three of them were gram-positive, while the other three were gram-negative (Table 1).

It is evident from Fig. 2 that *G. alkanivorans* CC-JG39, *R. erythropolis* CC-BC04 and *R. erythropolis* CC-BC11 tended to float and grow near the interface between the aqueous medium and diesel oil when they were cultivated in test tubes. In contrast, *C. testosteroni* CC-CF3, *S. yanoikuyae* CC-CG22, and *Acinetobacter* sp. CC-CF5 were evenly suspended in the medium under identical culture conditions (Fig. 2). As also demonstrated in Fig. 2c, after being grown in the diesel-containing medium for 10 d, the *G. alkanivorans* CC-JG39 cells moved to the oil-aqueous interface and formed small droplets (approximately 3.0–5.0 mm in diameter) entrapped by gel-like substances similar to air bags in structure.

Interestingly, the three isolates that were able to float are all gram-positive bacteria, whereas the nonfloating strains were all gram-negative (Table 1). Thus, it is intuitively considered that a difference in cell wall composition between gram-positive and -negative bacteria may give rise to the different floating behaviors of the isolates. However, as will be shown in the following section, we found that a gram-positive strain (*e.g.*, *Bacillus subtilis* CCRC16048) is not obligatorily a floating strain.

It was also suspected that the floating behavior might be driven by the demand of oxygen for cell growth (*i.e.*, aerotaxis effect). However, in our floating tests, bacterial cells were grown with shaking in test tubes for 4 d, before the test

<table>
<thead>
<tr>
<th>Strain</th>
<th>16S rDNA identity (standard strain)</th>
<th>Morphology</th>
<th>Colony color</th>
<th>Distribution in test tube</th>
<th>Gram staining</th>
<th>Utilizable substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gordonia alkanivorans</em> CC-JG39</td>
<td>1486/1488 (99%)</td>
<td>Rodlike</td>
<td>Orange</td>
<td>Floating</td>
<td>Positive</td>
<td>Diesel, benzene, toluene, xylene, naphthalene</td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em> CC-BC04</td>
<td>1342/1344 (99%)</td>
<td>Rodlike</td>
<td>White</td>
<td>Floating</td>
<td>Positive</td>
<td>Diesel, benzene, xylene</td>
</tr>
<tr>
<td><em>R. erythropolis</em> CC-BC11</td>
<td>1339/1341 (99%)</td>
<td>Rodlike</td>
<td>White</td>
<td>Floating</td>
<td>Positive</td>
<td>Diesel, benzene, toluene, ethylbenzene, xylene, naphthalene</td>
</tr>
<tr>
<td><em>Acinetobacter</em> sp. CC-CF5</td>
<td>387/394 (98%)</td>
<td>Spherical</td>
<td>White</td>
<td>Uniform</td>
<td>Negative</td>
<td>Diesel, benzene, toluene, ethylbenzene, xylene</td>
</tr>
<tr>
<td><em>Comamonas testosteroni</em> CC-CF3</td>
<td>1018/1018 (100%)</td>
<td>Rodlike</td>
<td>White</td>
<td>Uniform</td>
<td>Negative</td>
<td>Diesel, benzene, toluene, xylene</td>
</tr>
<tr>
<td><em>Sphingomonas yanoikuyae</em> CC-CG22</td>
<td>669/679 (98%)</td>
<td>Spherical</td>
<td>Brown</td>
<td>Uniform</td>
<td>Negative</td>
<td>Diesel, benzene, toluene, xylene, naphthalene</td>
</tr>
</tbody>
</table>

FIG. 1. (a) Growth curves and (b) residual diesel profiles of six bacterial isolates and a commercially available strain (*B. subtilis* CCRC16048) grown on BH medium supplemented with 0.1% diesel. Closed circles, Noninoculated medium; open circles, *G. alkanivorans* CC-JG39; solid reverse triangles, *R. erythropolis* CC-BC04; open reverse triangles, *R. erythropolis* CC-BC11; solid squares, *B. subtilis* BCRC16048; open squares, *C. testosteroni* CC-CF3; solid diamonds, *Acinetobacter* sp. CC-CF5; open diamonds, *S. yanoikuyae* CC-CG22.
tubes were allowed to stand for 24 h to observe the floating behavior. In these tests, whether bacteria are aerobic or facultatively anaerobic does not affect distribution in the culture medium because cells were grown under conditions with shaking. In fact, the floating strains found in this study (i.e., *Gordonia* and *Rhodococcus* species) were all nonmotile bacteria. Therefore, they cannot show aerotaxis; thereby, aerotaxis did not contribute to the floating activity of these actinobacteria.

**Quantitative analysis of floating activity of isolates**

The floating activity of the diesel-degrading strains was assessed quantitatively by determining the profiles of cell concentration distribution in the test tubes containing the mineral BH medium supplemented with 1% (v/v) diesel oil. Cell concentration distribution with respect to the depth from the medium interface (0, 1, 5 and 10 cm) is illustrated in Fig 3. It appears that nearly 55–70% of cells of the floating strains (CC-JG39, CC-BC04 and CC-BC11) shown in Fig. 2 accumulated near the oil-aqueous interface (Fig. 3a), whereas the nonfloating strains (CC-CF3, CC-CF5, and CC-CG22) evenly distributed in the tubes as shown in Fig. 3b. To determine whether all the gram-positive strains possess the floating ability in the oil-containing medium, an identical floating test was carried out for a commercially available gram-positive strain *B. subtilis* BCRC16048, which also utilizes diesel for growth. As shown in Figs. 2 and 3a, the *B. subtilis* cells did not show floating activity, but were suspended uniformly in the tube. Thus, gram positivity is not necessarily a requisite condition for floating activity.

**Cell wall distribution in sucrose density gradient**

Cell walls of the tested strains were isolated and subjected to sucrose density gradient centrifugation to identify whether the density of cell walls affected the floating activity. As shown in Fig. 4a, the cell wall fragments of the floating strains (CC-JG39, CC-BC04, and CC-BC11) essentially settled at low sucrose concentrations of 0% and 5%, whereas those of the nonfloating strains (CC-CF3, CC-CF5 and CC-CG22) distributed at a higher sucrose density (30%) (Fig. 4b). Thus, isolated cell walls of the three floating strains seem to have a lower density than those of cells unable to float. In a parallel experiment, intact cells were used for sucrose density gradient analysis. The results did not exhibit distinct trends for floating or nonfloating strains, as most of the cells located at the sucrose concentrations of 30–40% (data not shown).
The cell surface hydrophobicities of the six tested strains are shown in Table 2. It shows that *G. alkanivorans* CC-JG39 strain possessed the highest cellular hydrophobicities of 85% and 93% for kerosene and diesel, followed by CC-BC04 (12% and 21%) and CC-BC11 (13% and 15%), respectively. The cellular hydrophobicities of all non-floating strains (CC-CF3, CC-CF5, and CC-CG22) were very low (only 1–2% for CC-CF3 and CC-CG22 and less than 7% for CC-CF5). The results suggest that the strains with higher cell surface hydrophobicities float easily.

**Emulsification activity and surface tension reduction**

The hydrocarbon-utilizing bacteria often produce surface-active substances, such as biosurfactants (16, 17) or emulsifiers (18–20) to assist the uptake of hydrophobic substrates. Thus, the six diesel-degrading isolates were tested for their emulsification activities against diesel and kerosene and also for their ability to reduce the surface tension of water. As shown in Table 2, all the isolates showing floating activity (CC-JG39, CC-BC04, and CC-BC11) displayed poor emulsification activities of their supernatants, as the emulsion indexes ($E_{24}$) were all lower than 10%. In contrast, the supernatants of the strains with lower floating activities had higher emulsification abilities. The CC-CF3 and CC-CF5 strains exhibited 31% and 46% emulsification activity for kerosene, whereas the $E_{24}$ values decreased to 15% and 24% for diesel, respectively. The emulsification activity of cell residues exhibited opposite trends as compared with those for supernatants. The $E_{24}$ values for cell residues of strains CC-JG39, CC-BC04, and CC-BC11 were 2–3-fold higher than those of the supernatants. However, the cell residues of strains CC-CF3 and CC-CF5 possessed limited emulsification abilities for the two oils tested, as their $E_{24}$ values were lower than 10% for kerosene and 2% for diesel (Table 2). On the other hand, the surface tensions of the supernatants collected from the ESP culture of the six isolates ranged from 52.5 to 55.8 dyne/cm. This indicates an ineffective surface-tension-reducing activity, since the surface tensions of the cultures were similar to that of the pure LB medium (approximately 54.3 dyne/cm). Thus, the emulsification activities of the supernatants from CC-CF3 and CC-CF5 strains were believed to originate from their bioemulsifier products (such as proteinaceous substances [20]) that were ineffective in decreasing surface tension.

**SEM of *G. alkanivorans* CC-JG39**

Since *G. alkanivorans* CC-JG39 had the highest cellular hydrophobicity (Table 2) and floating activity among the six isolates studied, CC-JG39 cells were thus further analyzed by SEM to observe the variation of surface morphology at different times of culture. Figure 5a shows that cells from one-day-old cultures were clearly rod-like without visible formation of extracellular products, while those from 5-day-old cultures (Fig. 5b) were slightly shrunken, aggregated, and appeared to be covered by extracellular polymeric substances. It is thought that the extracellular products, probably the extracellular polymeric substances, may play a role in the floating behavior of *G. alkanivorans* CC-JG39. A preliminary analysis of the presence of EPS on *G. alkanivorans* CC-JG39 cells (from a 5-day-old culture) was performed using the Alcian blue 8GX staining technique (22), and the staining results indicated the presence of EPS on strain CC-JG39.
DISCUSSION

The data shown in Figs. 2 and 3 indicate that three of the six diesel-degrading isolates displayed floating behavior in the medium supplemented with diesel as the sole carbon source. Although it has been shown that not all the gram-positive strains can float (Fig. 3), the three floating strains examined in this study were all gram-positive, whereas the three gram-negative isolates were unable to float. Therefore, either the physical or chemical properties or both properties of the cell wall may be closely associated with the floating activity of the strains. Indeed, the sucrose density gradient analysis (Fig. 4) showed that the cell wall fraction of the floating strains settled at a lower sucrose density, while those of nonfloating strains distributed at a considerably higher sucrose density. This suggests that the density of cell wall components may be related to floating activity. In addition, the cellular hydrophobicity may also play an essential role in floating behavior (Table 2). All of the strains showing floating activity had much higher cellular hydrophobicities than those unable to float, particularly for G. alkani- vorans CC-JG39. Therefore, it is reasonable that the strains with higher cellular hydrophobicities tended to approach the hydrophobic carbon source (diesel) located on the upper surface of the medium, contributing to the floating behavior.

The other possible mechanism for floating is that the strains may secrete extracellular polymeric substances, such as exopolysaccharide biofilm (21, 23, 24), to enable the cells to float. Figure 2c shows that the cells of G. alkani- vorans CC-JG39 flocculated to form 3–5-mm-diameter droplets entrapped by gel-like substances, promoting the floating of cells probably due to a marked decrease in the bulk density of cells. The gel-like substance is most likely EPS as supported by the result of the Alcian blue staining. Quite recently, Fusconi and Godinho (21) have also reported the formation of EPS by a G. alkani- vorans strain. The results of SEM (Fig. 5) provided further evidence of the formation of extracellular products by G. alkani- vorans CC-JG39 after cultivation in the diesel-supplemented BH medium for 5 d. It is likely that EPS allow cells in proximity to adhere to each other forming a low-density network structure that facilitated cell floating. Once the cells approached the diesel layer on the surface, they accumulated near the diesel layer due to their high cellular hydrophobicity and also due to the need of carbon source for growth. EPS may facilitate the contact between bacterial cells and the oil substrate because it is also considered as an emulsifier (25). However, the detailed mechanism by which EPS contributes to the floating ability of G. alkani- vorans CC-JG39 cells certainly requires further investigation.
In this work, we observed that strains CC-JG39, CC-BC04, and CC-BC11 seemed to be capable of physically approaching the carbon source and stay there for further contact for their growth. This is evidenced by their floating activity (Figs. 2 and 3) as well as high cellular hydrophobicities (Table 2). The high emulsifying activities of their cell residues (Table 2) also indicate their ability for intimate contact with diesel oil, most likely due to the hydrophobic characteristics of the cell surface. The hydrophobicity might originate from their unique cell wall composition or from cell-wall-bound substances (such as EPSs in strain CC-JG39) produced in a hydrocarbon-rich environment. However, the production of extracellular emulsifiers was limited for the three floating strains as the supernatant from their cultures had low emulsification activities for diesel and kerosene (Table 2). In contrast, the nonfloating strains (such as CC-CF3 and CC-CF5) showed excellent supernatant emulsification activities (particularly for kerosene), it is likely that the ability to produce extracellular surface active substances for the emulsification of oil substrates might be their primary mechanism for the uptake and degradation of hydrophobic substrates.

The floating strains seemed to utilize diesel faster and more efficiently than most of the nonfloating strains, particularly at the early stage of growth (Fig. 1). This suggests the advantage of floating activity in the use of diesel for growth. However, as indicated in Fig. 1, some nonfloating strains (e.g., Acinetobacter sp. CC-CF5) may also be able to uptake diesel efficiently for rapid growth via other mechanisms, such as the secretion of an extracellular emulsifier that enhances the solubility of diesel.

In conclusion, due to the unique ability to move toward and attach to oil substrates that are usually located on the medium surface, the floating strains isolated in this work display a high potential for the bioremediation of oil-contaminated groundwater and marine systems. On the other hand, the isolates that could not float but produced more extracellular surface active substances may also be utilized to produce bioemulsifiers for oil bioremediation as well as for other commercial applications.

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