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Selective isolation of bacteria from soil with hydrophobic materials.

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Summary

Bacterial strains having a hydrophobic cell surface have often been considered as degraders of hydrophobic organic pollutants in soil. In this study, bacterial strains were isolated using hydrophobic materials from 12 soil samples, and their cell surface hydrophobicity was determined by evaluating their adherence to *n*-hexane. Bacterial strains isolated using polytetrafluoroethylene (PTFE) membrane were more hydrophobic on an average than those isolated with styrene-divinylbenzene (DVB) particles or octadecylsilyl silica gel (ODS) particles. Strains closely related to *Burkholderia cepacia* could be selectively isolated using the PTFE membrane; those closely related to *Ralstonia pickettii*, using ODS and DVB particles; and those closely related to *B. fungorum,* using DVB. These results indicate that bacterial strains having a hydrophobic cell surface or within certain phyla can be selectively isolated from soils using hydrophobic materials, and that this isolation method would be useful for collecting candidates for bioremediation of hydrophobic pollutants.

Keywords: *Burkholderia cepacia*; *Burkholderia fungorum*; cell surface hydrophobicity; octadecylsilyl silica gel; polytetrafluoroethylene; *Ralstonia pickettii*; selective isolation; styrene-divinyl benzene

Introduction

In general, hydrophobic organic compounds degrade slowly and persist for a long time in the environment. One of the reasons for their persistence is their low solubility in water and adsorption onto hydrophobic substances present in the environment, such as a part of hydrophobic humic substances, which results in low bioavailability and prevention of microbial attack (Tang et al. 1998).

Among the several characteristics of microorganisms utilized to overcome the low bioavailability of hydrophobic compounds, cell surface hydrophobicity is recognised as a factor responsible for their facilitated adhesion to hydrophobic interfaces such as the water-hydrocarbon interface and the surface of hydrophobic humic substances (Vacca et al. 2005; Colores et al. 2007). Obuekwe et al. (2009) found a significant correlation between cell surface hydrophobicity of bacterial strains capable of growing on crude oil and their ability to degrade it, although no correlation was reported for the microbial consortia (Owsianiak et al. 2009). Thus, methods for the selective isolation of bacteria having a hydrophobic cell surface would be useful for collecting candidates for the degradation of hydrophobic pollutants. Thus far, hydrophobic strains were isolated using hydrophobic materials such as Teflon, polysulphonate, Zirfon membranes, and acrylic ester resin (Neu and Poralla 1990; Bastiaens et al. 2000; Friedrich et al. 2000). In this study, bacterial strains were isolated from various soil samples using 3 types of hydrophobic materials, and their cell surface hydrophobicity was compared.

Materials and methods

Isolation of bacteria

Bacterial strains were isolated from 12 surface soil samples in Japan (Table 1). About 1.5 g of the soil sample was mixed with 30 ml of sterile water and the mixture was shaken vigorously. Then, 0.1 ml of the suspension was transferred to 5 ml of sterilized soil solution equivalent (SSE) medium (NH_4NO_3 0.2 g, K_2HPO_4 0.001 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCl_2$ 0.35 g, $NaNO_3$ 0.08 g, Fe $(NH_4)_2(SO_4)_2$ 0.006 g, KCl 0.036 g, distilled water 1 l) containing phenanthrene (approximately 50 mg l^{-1}). Phenanthrene was added into the SSE medium as crystals with no hydrophobic materials (NOM) or was held on either the PTFE membrane filter (3 μm pore size, Advantec Toyo, Japan), ODS particles (Wakogel 100C18, Wako, Japan), or DVB particles (Diaion HP20, Mitsubishi Chemical, Japan). The SSE medium enriched with the soil suspension was incubated at 28°C in dark conditions. After a couple of weeks, 0.05 ml of the SSE medium of NOM was transferred to 5 ml of a new SSE medium containing crystalline phenanthrene. For the SSE medium containing the PTFE membrane, the membrane was removed, washed with sterile water, and then transferred to another SSE medium containing a new PTFE membrane impregnated with phenanthrene. For the SSE medium containing ODS and DVB particles, the particles were washed with and resuspended in sterile water, and the suspension was mixed with a new SSE medium containing new ODS or DVB particles mixed with phenanthrene. After 3 such transfers, the SSE medium of NOM was serially diluted and then plated on the yeast extract glucose (YG) medium (K_2HPO_4 0.3 g, KH_2PO_4 0.2 g, $MgSO_4$ $7H_2O$ 0.2 g, glucose 1.0 g, yeast extract 1.0 g, agar 15 g, water 1 l). For PTFE, ODS, and DVB media, the membrane and the particles were transferred to

sterile water and subjected to ultrasonication (38kHz, 500W, IWAKI USC-400Z38S-24, Japan) for 1 min. The detached microbial suspension was serially diluted and then plated on the YG medium. Next, the plates were incubated at 28^{o}C in dark conditions. Bacterial colonies that grew on the plates were classified on the basis of their colony morphology, and representative 116 colonies were isolated after purification.

DNA extraction

Bacterial cells were lysed in Tris-EDTA (TE) buffer (10 mmol Tris, 1 mmol EDTA-2Na, water 1 l, pH 8.0) containing 0.2% sodium dodecyl sulphate. After standard purification with phenol and chloroform, DNA was precipitated with sodium acetate and ethanol, and then suspended in 20 μl of the TE buffer. DNA was successfully extracted from 106 isolates.

Polymerase chain reaction and temperature gradient gel electrophoresis (PCR-TGGE)

First, almost the entire 16S rRNA gene was amplified with primers 27f and 1492r (Osborne et al. 2005), and then the variable V3 region in the first PCR product was amplified using the forward primer 341f attached with a GC clamp at the 5' end and reverse primer 518r (Muyzer et al. 1993). Aliquots (4 μl) of the second PCR product were electrophoresed on 8% polyacrylamide gels (acrylamide/bis 37.5:1) containing urea (7 mol l^{-1}) and 28% formamide, in 1× Tris-acetate-EDTA (TAE) buffer (40 mmol Tris, 20 mmol acetic acid, 1 mmol EDTA-2Na, water 1 l, pH 8.0) at 90 V for 24 h. The ramp rate and temperature range were adjusted depending on the samples. After electrophoresis, the gels were stained with SYBR Green I for 30 min, and then viewed

under a transilluminater (480 nm). On the basis of the position of the amplified DNA, bacterial isolates were separated into groups.

Ribosomal intergenic spacer analysis (RISA)

Several isolates in each TGGE group were subjected to RISA analysis (Saito et al. 2007). The PCR primers used were ITSF and ITSReub (Cardinale et al. 2004). Aliquots (4 μl) of the PCR products were electrophoresed in 8% polyacrylamide gels (acrylamide/bis 37.5:1) containing urea (7.5 mol l^{-1}) in 1× TAE buffer at 180 V for 6 h. After staining with SYBR Green I, the gels were viewed under the transilluminater (480 nm). On the basis of the banding pattern of the DNA fragments, bacterial isolates were further separated into groups.

Sequencing of 16S rRNA genes

The 16S rRNA gene of several bacterial isolates was sequenced. Nearly the entire length of the 16S rRNA gene was sequenced by Bio Matrix Research Inc (Chiba, Japan). The sequences obtained were compared with those deposited in the DNA Data Bank of Japan (DDBJ)/the European Molecular Biology Laboratory (EMBL)/GenBank by using FASTA software (Pearson and Lipman 1988)(http://fasta.ddbj.nig.ac.jp/top-j.html), and the percent similarity was determined.

Measurement of cell surface hydrophobicity

Cell surface hydrophobicity of bacterial isolates was evaluated by bacterial adhesion to hydrocarbons (BATH)(Rosenberg et al. 1980; Donlon and Colleran 1993). In this study, bacterial cells grown in the YG medium at 28^{o}C in dark conditions were harvested by centrifugation (5,000 × *g*, 15 min), washed twice with phosphate urea magnesium (PUM) buffer (K_2PO_4 22.2 g, KH_2PO_4 7.26 g, urea 1.8 g, $MgSO_4$ $7H_2O$ 0.2 g, water 1 l, pH 7.2), and resuspended in PUM buffer to adjust its initial optical density (OD) (600 nm) to approximately 0.3. The bacterial suspension was mixed with an equal volume of *n*-hexane, vortexed for 2 min, and left for 20 min. Then, the OD of the aqueous phase was measured and its ratio to the initial OD was considered as cell surface hydrophilicity. Cell surface hydrophobicity was calculated using the following formula: (1 - cell surface hydrophilicity). Bacterial cells grown in triplicate were subjected to BATH. Statistical analysis was performed using the Student or Welch test at a level of P = 0.05.

Results and discussion

Among the 116 strains isolated, 106 strains were subjected to PCR-TGGE analysis. Based on the findings, 12 groups (Group I to XII) consisting of 87 strains and 19 unique strains (Table 2) were formed. Strains in Groups I to XII were further separated into 25 subgroups on the basis of the results of RISA.

Only 2 subgroups were obtained by isolation using ODS and DVB particles, whilst 15 subgroups were obtained by isolation using NOM. Strains belonging to *Ralstonia pickettii* were selectively isolated using DVB and ODS particles from 12 and 11 soil samples, respectively. DVB particles also selected the strains of *Burkholderia fungorum* from 12 soil samples. With the PTFE membrane, species related to *B. cepacia* (Groups I-1 to I-4, VIII-1) were isolated from 12 soil samples.

Cell surface hydrophobicity was determined for 74 strains. The average value of hydrophobicity of the PTFE isolates was higher than that for the NOM, ODS, or DVB isolates (Table 2). When compared within a certain phylogenetic range, the average value of cell surface hydrophobicity was higher again for the PTFE isolates. Within *Burkholderia* (Groups I, V-1, VI-1, VIII-1, and XII-1), the average hydrophobicity of the PTFE isolates was 43% (SD, 20%, n = 19), while that of the DVB and NOM isolates was 6% (SD, 2%, n = 5) and 11% (SD, 9 %, n = 8), respectively. Similarly, when compared within Group I-5 (species related to *B. caryophylli*), Group II-1 or Group IX, PTFE isolates were more hydrophobic.

Thus far, the isolation of hydrophobic strains with Teflon membrane has been reported (Bastiaens et al. 2000). The high hydrophobicity of PTFE isolates in the present results supports the previous finding; in fact, the PTFE membranes had a strong tendency to isolate hydrophobic strains. Cell surface hydrophobicity is assumed to be one of the

bacterial characteristics favouring adherence to hydrophobic interfaces such as hydrophobic pollutants and environmental sorbents (Vacca et al. 2005; Colores et al. 2007). Thus, isolation procedures using PTFE membranes would be useful in collecting hydrophobic bacterial strains, which are candidates for biodegradation of hydrophobic pollutants.

Although the results in this study showed that hydrophobic strains could be isolated using PTFE membranes, a part of hydrophobic strains might be lost during the isolation procedure. The use of sterile water for bacterial detachment may result in cell shock or even death, and the use of hydrophilic agar plates may decrease the number of hydrophobic species. Moreover, some isolates might change their cell surface hydrophobicity during their growth on the agar plates. In future, improvements in the isolation procedure that involve the use of hydrophobic materials may make it possible to obtain more diverse, hydrophobic strains from the soil.

This study also indicates that a certain phylogenetic group(s) can be selectively isolated using specific hydrophobic materials: *B. cepacia* with PTFE membrane; *B. fungorum,* with DVB particles; and *R. pickettii,* with ODS and DVB particles. It should be noticed that these bacterial groups were obtained from all or nearly all of the soil samples used in this study. These species are often considered good candidates for bioremediation because they contain strains capable of degrading organic pollutants (e.g., Coenye and Vandamme 2003; O'Sullivan and Mahenthiralingam 2005; Ryan et al. 2007). The present study reveals that PTFE, ODS, and DVB are suitable for the selective isolation of the respective species from soils.

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Table 1 Characteristics of the soil samples used in this study.

Soil	Type of land-use	Soil texture	Organic C (%)	pH (H_2O)
A	pasture	LiC	4.9	5.0
B	forest	LiC	6.4	5.2
C	forest	SiL	7.6	5.0
D	forest	LiC	15.4	4.2
E	forest	SiL	10.4	4.5
F	pasture	LiC	2.5	6.5
G	garden	SL	1.2	7.1
H	greenhouse	CL	4.2	6.5
I	forest	CL	5.7	4.8
J	agricultural field	CL	1.8	5.9
K	parking	S	0.3	6.8
L	parking	S	0.3	6.8

Table 2 Composition, cell surface hydrophobicity, and close relatives of the bacterial strains isolated with hydrophobic materials.

Group*	Isolation method†	No. of isolates	Hydrophobicity (%)‡			Closest 16S rDNA sequence in DDBJ/EMBL/GenBank		
			Mean	SD or deviation	n	Accession No.	Taxa	Similarity (%)
I-1	PTFE	7	56	11	7	EU214612	*Burkholderia cepacia*	99.8
I-2	PTFE	7	36	18	7	CP000379	*B. cenocepacia*	99.9
I-3	NOM	1	33		1	AB041730	*Burkholderia* sp.	99.6
I-4	PTFE	3	28	10	2	AY741349	*B. cepacia*	99.0
I-5	NOM	2	10	0	2	AF408965	*Burkholderia* sp.	97.2
	PTFE	1	30		1			
I-6	NOM	1	10		1	AF508806	*Burkholderia* sp.	98.9
II-1	NOM	1	8		1			
	PTFE	2	32	1	2			
III-1	NOM	2	5	3	2			
IV-1	NOM	1	11		1	DQ232614	*Leifsonia shinshuensis*	99.2
IV-2	NOM	1			0			
V-1	NOM	1	13		1	AY238506	*Burkholderia* sp.	92.5
V-2	NOM	1	7		1			
V-3	NOM	1	28		1			
V-4	PTFE	1	8		1			
VI-1	NOM	3	9	5	3	CP000271	*B. xenovorans*	99.5
VII-1	NOM	1	3		1			
VII-2	NOM	1			0			
VII-3	NOM	1	7		1			
VII-4	PTFE	1	11		1			
VII-5	PTFE	1	11		1			
VIII-1	PTFE	2	61	21	2	CP000458	*B. cenocepacia*	99.6
IX	NOM	1	4		1			
	PTFE	1	46		1			
X-1	DVB	12	7	3	5	AJ270260	*Ralstonia pickettii*	99.9
	ODS	11	8	1	3			
XI-1	ODS	7	8	6	4			
XII-1	DVB	12	6	2	5	AJ544690	*B. fungorum*	99.8
others	NOM	12	28	27	10			
	DVB	2			0			
	ODS	9	43	20	2			
	PTFE	6	16	9	3			
total	NOM	31	17	19	27			
	DVB	26	6	2	10			
	ODS	27	16	18	9			
	PTFE	32	37	20	28			

*Based on results of PCR-TGGE of V3 in 16S rRNA gene and RISA.

† PTFE, isolated using polytetrafluoroehylene membrane; DVB, using divinylbenzene particle; ODS, using octadecylsilyl silica gel; NOM, without hydrophobic materials.

‡ Bacterial adherence to *n*-hexane.