

Full Paper

Phylogenic analysis of bacteria passed through 0.45- μ m-pore-size filters in the rhizosphere

Yosuke Tabei and Kohji Ueno*

The Laboratory of Microbiology, Faculty of Pharmacy, Research Institute for Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Musashino University, Nishitokyo 202-8585, Tokyo, Japan

(Received August 6, 2009; Accepted November 5, 2009)

Molecular analyses of 16S ribosomal RNA genes (rDNA) revealed that microbial communities in the rhizosphere are highly complex. To systematically characterize the cell size of the bacteria in the rhizosphere, we selected bacteria that potentially could be passed through 0.22- or 0.45- μ m-pore-size filters and then PCR amplified the 16S rDNA genes using the universal primer pairs 27F/1492R and 63F/1387R. The PCR-amplified rDNAs extracted from bacteria that had been passed through 0.45- μ m-pore-size filters could be detected in agarose gels after electrophoresis; whereas after filtration of the bacteria through 0.22- μ m-pore-size filters no PCR-amplified rDNAs were found. Comparison of random cloning and sequencing of the libraries of the PCR-amplified rDNAs with or without cell size selection showed that bacteria belonging to the candidate phylogenic divisions of OD1 (OP11-derived 1), OP11, TM7, and OP5 can be concentrated by cell size selection using 0.45- μ m-pore-size filters, but not by using 0.22- μ m-pore-size filters. OD1, OP11, TM7 and OP5 bacteria have yet to be cultivated; therefore, our concentration method may be used as an initial step in studies to analyze the structural properties of OD1, OP11, and TM7 bacteria.

Key Words—filterable bacteria; rhizosphere; 16S rRNA genes

Introduction

Molecular analyses of 16S rRNA gene cloning and sequencing methods were applied to identify microorganisms in environmental samples including, soil, seawater, and wastewater. The analyses revealed that

the diversity of bacterial communities in the soil is highly complex and the soil contains bacteria which belong to the candidate phylogenic divisions (Rappé and Giovannoni, 2003). The candidate phylogenic divisions are known as the phylogenic divisions which did not contain cultivated relatives. The properties of the bacteria in the candidate phylogenic divisions have yet to be characterized because cultivation conditions for the bacteria have not yet been established.

The rhizosphere is defined as the soil surrounding the roots that is influenced by living roots. This influence may occur by root exudation of carbon substrates that affect microbial communities. Therefore the rhizosphere is one good source for the analysis of the microbial communities of the soil because the soil of the rhizosphere contains relatively abundant bacteria. In the rhizosphere, it is well known that highly diverse of

* Address reprint requests to: Dr. Kohji Ueno, The Laboratory of Microbiology, Faculty of Pharmacy, Research Institute for Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi, Nishitokyo 202-8585, Tokyo, Japan.

Tel & Fax: 81-42-468-8645

E-mail: kueno@musashino-u.ac.jp

The novel sequences reported in this paper are available through the DDBJ, EMBL, and GenBank, accession numbers AB510984-AB511020.

bacteria exist (Kent and Triplett, 2002).

Small bacteria that can be filtered through 0.2- or 0.45- μm -pore-size filters have been found in environmental samples including soil, seawater, and deep ground water (Hahn et al., 2003; Iizuka et al., 1998; Miyoshi et al., 2005). In research laboratories, 0.22- and 0.45- μm -pore-size filters are commonly used to remove bacteria from fluids. To structurally characterize the bacteria in the rhizosphere, we systematically examined cell size selection of rhizosphere bacteria using 0.22- and 0.45- μm -pore-size filters and performed identification of the bacteria by rDNA analysis.

In this paper, we found that we can concentrate OD1, OP11, TM7, and OP5 bacteria from soil of the rhizosphere using 0.45- μm -pore-size filters, but not by using 0.22- μm -pore-size filters. TM7 bacteria were first found in a German peat bog (Torf, mittlere Schicht, or peat, middle layer) (Rheims et al., 1996) and have since been detected in other environmental samples including, soil, seawater, and wastewater (Hugenholtz et al., 1998, 2001). OP11 and OP5 bacteria were found in the Yellowstone hot spring Obsidian Pool (Hugenholtz et al., 1998). OD1 bacteria were found in other environmental samples (Harris et al., 2004). OD1, OP11, TM7, and OP5 bacteria belong to the candidate phylogenetic divisions. No mechanisms for cultivation of OD1, OP11, TM7 or OP5 bacteria are available, therefore the structural properties of OD1, OP11, TM7 and OP5 bacteria have yet to be characterized. Our method for concentrating OD1, OP11, TM7 and OP5 bacteria from the rhizosphere may be useful as the initial step in studies to characterize the structural properties of the bacteria without culture.

Materials and Methods

Sample collection and the preparation of the bacterial extracts. We collected rhizosphere samples of a daisy which was bought from a flower shop. Five grams of rhizosphere and soil samples around roots in plastic flower pots were suspended in 5 ml of phosphate-buffered saline (PBS) in 50-ml tubes and the tubes were agitated at 2,000 rpm for 3 min at room temperature using a vortex mixer (TAITEC S-100, Japan). After standing for 5 min, the suspension was collected and centrifuged at 300 rpm for 1 min to remove insoluble substances using a MX-300 centrifuge (approximately $10 \times g$, TOMY Co., LTD, Japan). After the resulting supernatant was centrifuged at $20,000 \times g$

for 10 min, the pelleted bacteria were suspended in 100 μl of PBS, containing 0.05% Tween 80. After agitation of the tube for 10 s, 400 μl of PBS was added. The suspension was agitated at 1,000 rpm using a TAITEC BR-25 Apparatus (Japan) for 3 min at 4°C and centrifuged at 300 rpm for 1 min in a MX-300 centrifuge.

We prepared bacterial DNA from 400 μl of the supernatant of rhizosphere samples without including the filtration step. DNA was extracted using the Ultra-CleanTM microbial DNA isolation kit (MO BIO Laboratories, Inc., Japan).

Four hundred microliters of the supernatant prepared from 5 g of rhizosphere and soil samples underwent cell size selection using filters. Two hundred microliter samples of the supernatant were then gently and carefully passed through a 0.22- or 0.45- μm -pore-size filter (Millex[®] 13 mm diameter syringe filter unit (catalog number SLHV013SL or SLGV013SL, Millipore, USA) with pressure applied via a 1-ml disposable syringe. The filtrated microorganisms were heated at 95°C for 3 min immediately after filtration and then centrifuged at $20,000 \times g$ for 10 min. The pelleted bacteria were suspended in 500 μl of PBS and centrifuged at $20,000 \times g$ for 10 min. The pellets were then washed twice. The final pelleted bacteria were suspended in 10 μl 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.5% Triton X-100, and were then disrupted by sonication for 2 min using a Bioruptor UCW-201 (Cosmo Bio Co., LTD, Japan) at a power setting of 80 W. The bacterial extracts were subjected to PCR amplification.

Amplification of 16S rDNA by PCR. We used two sets of universal primer pairs to amplify the bacterial 16S rDNA: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTACGACTT-3') (Lane, 1991); and 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWTGTACAAGGC-3') (Marchesi et al., 1998). Each amplification was performed in a total volume of 25 μl containing 0.25–0.5 μl of the bacterial extract, 5 pmol of each primer, 200 μM dNTP, 1.25 U of Gene Taq FP DNA polymerase (Nippon Gene Co., LTD, Japan), and 1.25 U of Taq ExtenderTM PCR additive (Stratagene, USA) in Taq ExtenderTM Reaction Buffer. PCR reactions were performed using a Perkin-Elmer 9700 Thermocycler. The reaction conditions were 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s, followed by a final extension at 72°C for 5 min. Three microliters of the amplification mixture was electrophoresed on a 1.2% agarose gel. The DNA was stained with ethidium bromide

and then visualized under short-wavelength UV light.

We used *Taq* Extender™ PCR additive and Gene *Taq* FP DNA polymerase for PCR amplifications of the 16S rDNA of bacteria that passed through 0.22- or 0.45- μ m-pore-size filters. As we obtained only small amounts of the PCR products when *Taq* Extender™ PCR additive was not used, it was a necessary addition. Gene *Taq* FP DNA polymerase is highly purified from contaminated bacterial DNA. In the absence of template DNA, no PCR-amplified 16S rDNA could be detected.

Cloning procedure. PCR products were purified using PureLink™ PCR Purification Kit reagents (Invitrogen, USA) and concentrated with SUPREC®-PCR spin columns (TaKaRa, Japan). Cloning of the purified DNA was performed with pGEM®-T Easy Vector System (Promega, USA) reagents according to the manufacturer's instructions. Transformation was done with competent *Escherichia coli* JM109 cells provided by TaKaRa. The transformed cells were plated onto Luria-Bertani agar supplemented with ampicillin and incubated overnight at 37°C. Each colony was placed into 25 μ l of *TaKaRa Ex Taq*® Hot Start Version (TaKaRa) and PCR was then performed using the forward primer (2949–2972) and the reverse primer (174–197). The sizes of the inserts were determined by electrophoresis using a 1.2% agarose gel.

DNA sequencing and phylogenetic analysis. Prior to sequencing the PCR-amplified 16S rDNA fragments, they were purified and concentrated using SUPREC®-PCR spin columns (TaKaRa). The dideoxy chain termination reaction was performed in both directions using the PCR-amplified 16S rDNAs with forward and reverse primers. The products were analyzed using an ABI PRISM 3730XL apparatus (Applied Biosystems, USA).

To identify their closest relatives, the sequences were compared to the 16S rDNA sequences in the Ribosomal Database Project (Cole et al., 2009). The nucleotide sequences were also characterized by a BLASTN search (GenBank) for the nearest matches. The sequences were assessed using the Chimera Check program (Ribosomal Database Project II) (Cole et al., 2003). The cutoff for species differentiation was 2%, which equates to approximately 30 bases for a full sequence. We defined a phylotype as a cluster of cloned sequences that differed from those of known species by more than 2%, yet had at least 98% internal sequence similarities among the members of the cluster (Suau et al., 1999). The phylotypes that corre-

sponded to sequences found in the Ribosomal Database were labeled as known phylotypes, whereas those that did not correspond to any in the database were labeled as novel phylotypes.

Sequence alignments were performed, and a phylogenetic tree was constructed using the neighbor-joining algorithm of ARB software (Ludwig et al., 2004). The 16S rRNA sequence of *Escherichia coli* and *Shewanella putrefaciens* were used as out-groups to root the tree. Construction of the tree used the bootstrap method and 100 replicate samples of the dataset (Felsenstein, 1985).

Nucleotide sequence accession numbers. The novel sequence data determined in this study have been assigned DDBJ, EMBL, and GenBank accession numbers AB510984–AB511020.

Results and Discussion

PCR analyses of filterable bacteria in the rhizosphere

We characterized the PCR-amplified rDNA obtained from rhizosphere bacteria that had been passed through 0.22- or 0.45- μ m-pore-size filters. Figure 1 (lanes 1 and 2) shows PCR products synthesized in the presence of the primer pair 27F/1492R. The PCR products obtained from the extracts of bacteria that had been passed through 0.45- μ m-pore-size filters

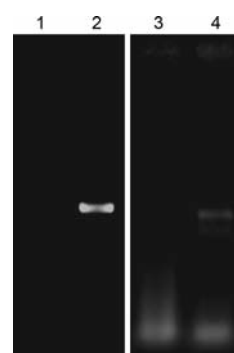


Fig. 1. PCR analysis of the rhizosphere bacteria that passed through 0.22- or 0.45- μ m-pore-size filters.

We performed PCR reactions with 27F/1492R (lanes 1 and 2) or 63F/1387R primer pairs (lanes 3 and 4). Samples obtained from the filtrate that had been filtered through 0.22- μ m-pore-size filters are shown in odd-numbered lanes. Those obtained from bacteria that had been filtered through 0.45- μ m-pore-size filters are shown in even-numbered lanes. Each 3- μ l sample of the PCR amplification reaction mixture was examined by 1.2% agarose gel electrophoresis. The amplified DNA fragments were approximately 1,500 base pairs (lane 2) and approximately 1,300 base pairs (lane 4).

and had been obtained from rhizosphere are shown in lane 2. However, no PCR product was detected in the filtrate which had been filtered through 0.22- μ m-pore-size filters (lane 1). Using the primer pair 63F/1387R (lanes 3 and 4 of Fig. 1), a small amount of PCR product was seen when the bacteria had been passed through 0.45- μ m-pore-size filters (lane 4), but no PCR product was detected in the filtrate which had been filtered through 0.22- μ m-pore-size filters (lane 3). We examined the PCR analysis of 16 rhizosphere samples with the 2 sets of universal primer pairs. We obtained PCR products from 6 rhizosphere samples but no or only small amounts of products from 10 rhizosphere samples.

We performed heat treatment of the filterable samples at 95°C immediately after 0.45- μ m-pore-size filtration. We also performed two washing procedures after centrifugation of the heat-treated samples to obtain sufficient amounts of PCR products for cloning and sequence analyses. We speculated that the rhizosphere may contain an inhibitor for PCR amplification.

The PCR amplifications were performed with 0.5 μ l of the bacterial extracts that had been passed through 0.45- μ m-pore-size filters as shown in Fig. 1. Greater amounts of extracts reduced the amounts of PCR products. As we could not obtain sufficient PCR products with the primer pair 63F/1387R, we analyzed the PCR products that were amplified with the primer pair 27F/1492R for the cloning and sequence.

Sequence analyses of the rDNAs of the filterable bacteria obtained from rhizosphere

We randomly isolated a total of 84 clones from two libraries that had been constructed using the PCR-amplified rDNAs obtained by amplification with the primer pair 27F/1492R with filtered bacteria. We also randomly isolated a total of 81 clones from two libraries that were constructed from the PCR-amplified products from the DNA extracts of rhizosphere bacteria without using cell size selection. Analyses of the rDNA sequences using the Ribosome Database Project and ARB phylogenetic analysis software revealed that the clones were derived from several bacterial clusters, as shown in Table 1. More clones of OD1, OP11, TM7, and OP5 and *Deltaproteobacteria* were obtained using size selection than without size selection. None of the OD1 bacteria was detected in a total of 81 clones prepared without size selection, whereas 6 clones were detected in a total of 84 clones with fil-

Table 1. Number of rhizosphere clones with or without size selection.

	without filtration		with filtration	
	lot. (a)	lot. (b)	lot. (a)	lot. (b)
phylum <i>Proteobacteria</i>				
class <i>Alphaproteobacteria</i>				
order <i>Caulobacteriales</i>	2	1		
order <i>Rhizobiales</i>	6	4		1
unclassified <i>Alphaproteobacteria</i>		1		2
class <i>Betaproteobacteria</i>				
order <i>Burkholderiales</i>	8	6		3
order <i>Rhodocyclales</i>	1	1		
unclassified <i>Betaproteobacteria</i>	2	3	1	8
class <i>Gammaproteobacteria</i>				
order <i>Xanthomonadales</i>	9	3	2	3
order <i>Legionellales</i>	1	3	2	1
order <i>Oceanospirillaceae</i>				1
unclassified <i>Gammaproteobacteria</i>	3		2	
class <i>Deltaproteobacteria</i>				
order <i>Bdellovibrionales</i>				
family <i>Bdellovibrionaceae</i>				
genus <i>Bdellovibrio</i>	1		2	3
unclassified <i>Deltaproteobacteria</i>		2	5	4
family <i>Bacteriovoracaceae</i>				
genus <i>Peredibacter</i>				1
genus <i>Bacteriovorax</i>			1	
unclassified <i>proteobacteria</i>	1			
phylum <i>Actinobacteria</i>				
class <i>Actinobacteria</i>				
order <i>Acidimicrobiales</i>				1
phylum <i>Acidobacteria</i>				
class <i>Acidobacteria</i>				
order <i>Acidobacteriales</i>		2	1	
phylum <i>Firmicutes</i>				
class <i>Bacilli</i>				
order <i>Bacillales</i>	1			
phylum <i>Planctomycetes</i>				
class <i>Planctomycetacia</i>				
order <i>Planctomycetales</i>	1	1		
phylum <i>Bacteroidetes</i>				
class <i>Sphingobacteria</i>				
order <i>Sphingobacteriales</i>	1	4		
phylum <i>Verrucomicrobia</i>				
class <i>Verrucomibiae</i>				
order <i>Verrucomicrobiales</i>		3		
phylum <i>Gemmatimonadetes</i>				
class <i>Gemmatimonadates</i>				
order <i>Gemmatimonadales</i>	1			
phylum OP10		1		
phylum OD1			2	4
phylum OP11	1		7	2
phylum TM7		1	13	4
phylum OP5			2	3
unclassified <i>Bacteria</i>	2	4		1
total	41	40	42	42

tered bacteria. One clone of OP11 bacteria was detected in a total of 81 clones without size selection, whereas a total of 9 clones were detected in a total of 84 clones with filtered bacteria. One clone of TM7 bacteria was detected in a total of 81 clones without size selection, whereas 17 clones were detected in 84 clones with filtered bacteria.

OD1, OP11, and TM7 bacteria in the rhizosphere

The ARB analysis showed one cluster of bacteria belonging to the OD1, OP11, and TM7 phylogenetic divisions. The sequences of 32 clones of a total of 84 clones from two lots of rhizosphere samples using size selection were derived from OD1, OP11, and TM7 bacteria. Figure 2 shows a phylogenetic tree of OD1, OP11, and TM7 bacteria. A total of 32 clones belonging to 6 phylotypes of OD1, 8 phylotypes of OP11, and 11 phylotypes of TM7 were identified. As shown in Table 2, only one of the 25 phylotypes of OD1, OP11, and TM7 bacteria was a known phylotype. The other 24 phylotypes were novel phylotypes with less than 98% sequence similarity to known sequences. As shown in Table 2, the sequence similarities of novel phylotypes of TM7 bacteria are 0.93–0.97 to those of known phylotypes. The sequence similarities of OD1 and OP11 novel phylotypes were 0.81–0.95 and 0.87–0.93, respectively, to those of known phylotypes. The low similarity of OD1 and OP11 bacteria reflect greater evolutionary distances than TM7 bacteria, as shown in Fig. 2.

From these results, we suggest that OD1, OP11, and TM7 bacteria present in the rhizosphere sample are concentrated by filtration through 0.45- μ m-pore-size filters. So far there is no information about the size of TM7 bacteria in the rhizosphere. According to the work of Miyoshi et al. (2005), OD1 and OP11 bacteria passed through 0.2- μ m-pore-size filters and were captured by 0.1- μ m-pore-size filters in deep ground water (approximately 200 m below ground level). This work suggested that OD1 and OP11 bacteria in deep ground water may be smaller than 0.2 μ m.

As cultivation conditions for OD1, OP11 and TM7 bacteria have not yet been established, the structural properties of OD1, OP11, and TM7 bacteria have not been well characterized. However, our finding that OD1, OP11, and TM7 bacteria can be concentrated after filtration through a 0.45- μ m-pore-size filter is a new concentration method and may be a useful initial step for characterization of OD1, OP11, and TM7 bac-

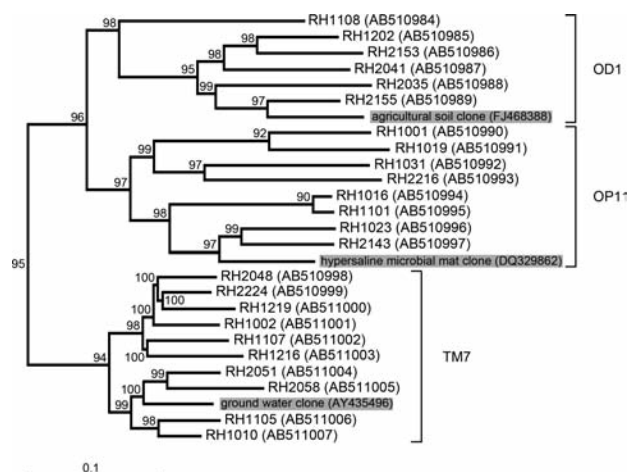


Fig. 2. Phylogenetic analyses of the rhizosphere OD1, OP11, and TM7 bacteria that passed through 0.45- μ m-pore-size filters.

The tree was constructed by neighbor-joining using ARB software. The 16S rRNA sequence of *E. coli* and *S. putrefaciens* were used as out-groups to root the tree. Novel phylotypes detected in this study are named with the prefix RH (for rhizosphere). Accession numbers are given in parentheses. The known phylotype is highlighted in gray. The bars represent evolutionary distances. Bootstrap values are shown.

teria.

Deltaproteobacteria in the rhizosphere

Sixteen clones of 13 phylotypes of subclass *Deltaproteobacteria* are shown in Fig. 3. Two of 13 phylotypes of *Deltaproteobacteria* were known phylotypes; the other 11 phylotypes were novel phylotypes with less than 98% similarity to known phylotypes (Table 2).

Five clones of the filtered bacteria of subclass *Deltaproteobacteria* are *Bdellovibrio* species, as shown in Fig. 3 and Table 2. One of the most notable characteristics of *Bdellovibrio* sp. is that members parasitize other Gram-negative bacteria by entering the periplasmic space and feeding on the proteins and nucleic acids of their hosts. *Bdellovibrio* are comma-shaped motile rod bacteria that are about 0.3 by 0.5 μ m in size (Stolp and Strarr, 1963). Owing to the bacterial cell size, we speculated that we can concentrate *Bdellovibrio* sp. by 0.45- μ m-pore-size filtration.

Nine clones of the filtered bacteria of *Deltaproteobacteria* belong to seven phylotypes of unclassified *Deltaproteobacteria*, as shown in Fig. 3 and Table 2. The sequence of FJ479048 from undisturbed grass prairie was submitted to GenBank (not published).

Table 2. The clones of filterable OD1, OP11, TM7, *Deltaproteobacteria*, and OP5 bacteria from the rhizosphere.

Phylum	Name	Accession No.	Number of clones		Nearest	Similarity
			lot. (a)	lot. (b)		
OD1	RH1108	AB510984	1	0	FJ482179	0.81
	RH1202	AB510985	1	0	EF516449	0.92
	RH2153	AB510986	0	1	EF516850	0.95
	RH2041	AB510987	0	1	AY850299	0.90
	RH2035	AB510988	0	1	EF018926	0.88
	RH2155	AB510989	0	1	FJ468388	0.89
OP11	RH1001	AB510990	1	0	AB179670	0.89
	RH1019	AB510991	1	0	AB179670	0.92
	RH1031	AB510992	1	0	EU385901	0.87
	RH2216	AB510993	0	1	FJ482190	0.93
	RH1016	AB510994	2	0	EF444716	0.85
	RH1101	AB510995	1	0	EF444716	0.85
	RH1023	AB510996	1	0	AY667254	0.87
	RH2143	AB510997	0	1	DQ329862	0.87
TM7	RH2048	AB510998	0	1	EF016808	0.93
	RH2224	AB510999	0	1	AM991156	0.97
	RH1219	AB511000	1	0	AY345503	0.95
	RH1002	AB511001	5	0	EU800550	0.97
	RH1107	AB511002	1	0	AM991156	0.93
	RH1216	AB511003	1	0	EU135364	0.94
	RH2051	AB511004	1	1	AF525832	0.96
	RH2058	AB511005	0	1	EU104291	0.94
	ground water clone	AY435496	2	0		
	RH1105	AB511006	1	0	EU135369	0.94
	RH1010	AB511007	1	0	EU861856	0.93
<i>Deltaproteobacteria</i>	mud clone	EU431712	0	1		
	RH2049	AB511008	0	1	EU431712	0.97
	RH2138	AB511009	0	1	AF148938	0.97
	RH1003	AB511010	2	0	EF516103	0.95
	RH1006	AB511011	1	0	FJ479048	0.96
	RH1015	AB511012	1	0	FJ479048	0.96
	RH1020	AB511013	2	0	FJ479048	0.97
	RH1118	AB511014	1	0	FJ479048	0.97
	grass clone	FJ479048	0	1		
	RH2151	AB511015	0	1	FJ479048	0.97
	RH2156	AB511016	0	2	FJ479048	0.97
	RH2201	AB511017	0	1	AY294222	0.90
	RH1032	AB511018	1	0	AF030781	0.91
OP5	RH2150	AB511019	1	2	AY133074	0.97
	RH1211	AB511020	1	0	AY133074	0.97
	TCE-contaminated site clone	AY133074	0	1		

OP5 bacteria in the rhizosphere

One clone of OP5 bacteria was detected in a total of 81 clones without size selection, whereas 5 clones were detected in 84 clones with filtered bacteria. These

five clones belonged to three phylotypes of OP5 bacteria, as shown in Fig. 4 and Table 2. Two phylotypes were novel phylotypes with less than 98% similarity to known phylotypes (Table 2). In Fig. 4, *Caldiesericum*

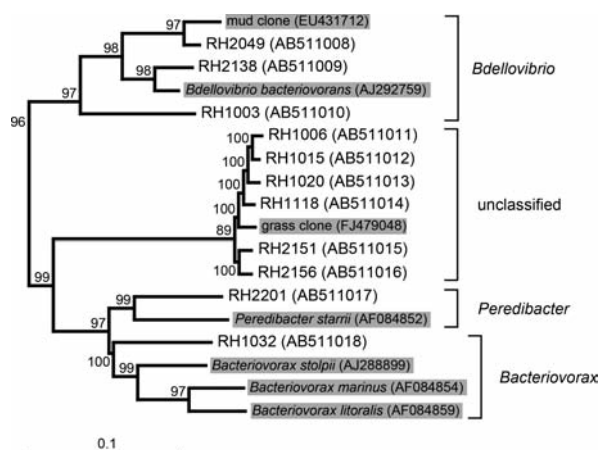


Fig. 3. Phylogenetic analyses of the rhizosphere *Deltaproteobacteria* that passed through 0.45- μ m-pore-size filters.

The tree was constructed by neighbor-joining using ARB software. The 16S rRNA sequence of *E. coli* and *S. putrefaciens* were used as out-groups to root the tree. Novel phylotypes detected in this study are named with the prefix RH (for rhizosphere). Accession numbers are given in parentheses. The known phylotypes are highlighted in gray. The bars represent evolutionary distances. Bootstrap values are shown.

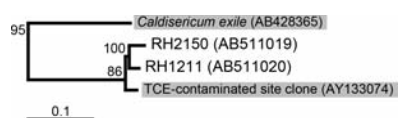


Fig. 4. Phylogenetic analyses of the rhizosphere OP5 bacteria that passed through 0.45- μ m-pore-size filters.

The tree was constructed by neighbor-joining using ARB software. The 16S rRNA sequence of *E. coli* and *S. putrefaciens* were used as out-groups to root the tree. Novel phylotypes detected in this study are named with the prefix RH (for rhizosphere). Accession numbers are given in parentheses. The known phylotype is highlighted in gray. The bars represent evolutionary distances. Bootstrap values are shown.

exile AZM16c01, which belongs to the OP5 phylum and recently proposed new phylum *Caldirserica*, was included (Mori et al., 2008, 2009).

Acknowledgments

This work was supported by MEXT. HAITEC (2004–2008). We are grateful to Mrs T. Kitada for technical support.

References

Cole, J. R., Chai, B., Marsh, T. L., Farris, R. J., Wang, Q., Kulam, S. A., Chandra, S., McGarrell, D. M., Schmidt, T. M., Garrity, G. M., and Tiedje, J. M. (2003) The Ribosomal Database Project (RDP-II): Previewing a new autoaligner that allows

regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.*, **31**, 442–443.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M., and Tiedje, J. M. (2009) The Ribosomal Database Project: Improved alignment and new tools for rRNA analysis. *Nucleic Acids Res.*, **37**, 141–145.

Felsenstein, J. (1985) Confidence limits of phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791.

Hahn, M. W., Lünsdorf, H., Wu, Q., Schauer, M., Höfle, M. G., Boenigk, J., and Stadler, P. (2003) Isolation of novel ultramicrobacteria classified as *Actinobacteria* from five freshwater habitats in Europe and Asia. *Appl. Environ. Microbiol.*, **69**, 1442–1451.

Harris, J. K., Kelley, S. T., and Pace, N. R. (2004) New perspective on uncultured bacterial phylogenetic division OP11. *Appl. Environ. Microbiol.*, **70**, 845–849.

Hugenholtz, P., Brett, M. T., and Pace, N. R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.*, **180**, 4765–4774.

Hugenholtz, P., Tyson, G. W., Webb, R., Wagner, A. M., and Blackall, L. L. (2001) Investigation of candidate division TM7, a recently recognized major lineage of the domain *Bacteria* with no known pure-culture representatives. *Appl. Environ. Microbiol.*, **67**, 411–419.

Iizuka, T., Yamanaka, S., Nishiyama, T., and Hiraishi, A. (1998) Isolation and phylogenetic analysis of aerobic copiotrophic ultramicrobacteria from urban soil. *J. Gen. Appl. Microbiol.*, **44**, 75–84.

Kent, A. D. and Triplett, E. W. (2002) Microbial communities and their interaction in soil and rhizosphere ecosystem. *Annu. Rev. Microbiol.*, **56**, 211–236.

Lane, D. J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, ed. by Stackebrandt, E. and Goodfellow, M., Blackwell, Oxford, UK, pp. 115–175.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K. H. (2004) ARB: A software environment for sequence data. *Nucleic Acids Res.*, **32**, 1363–1371.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., and Wade, W. G. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.*, **64**, 795–799.

Miyoshi, T., Iwatsuki, T., and Naganuma, T. (2005) Phylogenetic characterization of 16S rRNA gene clones from deep-groundwater microorganisms that pass through 0.2-micrometer-pore-size filters. *Appl. Environ. Microbiol.*, **71**,

- 1084–1088.
- Mori, K., Sunamura, M., Yanagawa, K., Ishibashi, J., Miyoshi, Y., Iino, T., Suzuki, K., and Urabe, T. (2008) First cultivation and ecological investigation of a bacterium affiliated with the candidate phylum OP5 from hot spring. *Appl. Environ. Microbiol.*, **74**, 6223–6229.
- Mori, K., Yamaguchi, K., Sakiyama, Y., Urabe, T., and Suzuki, K. (2009) *Caldisericum exile* gen. nov., sp. nov., an anaerobic thermophilic, filamentous bacterium of a novel bacterial phylum, *Caldiserica* phyl. nov., originally called the candidate phylum OP5, and description of *Caldiseriaceae* fam. nov., *Caldisericales* ord. nov. and *Caldisericia* classis nov. *Int. J. Syst. Evol. Microbiol.*, **59**, 2894–2898.
- Rappé, M. S. and Giovannoni, S. J. (2003) The uncultured microbial majority. *Annu. Rev. Microbiol.*, **57**, 369–394.
- Rheims, H., Spröer, C., Rainey, F. A., and Stackebrandt, E. (1996) Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations. *Microbiology*, **142**, 2863–2870.
- Suau, A., Bonnet, R., Sutren, M., Godon, J. J., Gibson, G. R., Collins, M. D., and Doré, J. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.*, **65**, 4799–4807.
- Stolp, H. and Strarr, M. P. (1963) *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. *Antonie van Leeuwenhoek*, **29**, 217–248.