Short Communication

Isolation and characterization of a new aromatic compound-degrading alkalitrophic bacteria

Michihisa Maeda,^{1,2} Michael S. Roberts,^{1,3} Yoshinori Ohta,^{1,2} Fumie Fuji,^{1,2}
Michael Travisano,^{1,3} and Toshiaki Kudo^{1,2,*}

¹Institute of Physical and Chemical Research (RIKEN) and

²Research Development Corporation of Japan (JRDC), Wako 351–0198, Japan

³Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824–13253, U.S.A.

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Aromatic compounds such as polychlorinated biphenyls (PCBs) are serious environmental pollutants throughout the world. It has been shown that biphenyl-utilizing bacteria are distributed in the environment and can cometabolize many PCB congeners to chlorobenzoic acids via oxidative pathways (Asturias and Timmis, 1993; Bedard et al., 1986, 1987; Furukawa et al., 1979; Mondello, 1989). Since lignin contains biphenyl moieties or aromatic compounds, we anticipated that an aromatic compound such as biphenyl/PCB-degrading bacteria might be present in natural habitats and isolated a number of aromatic compound-degrading bacteria.

Some chemical pretreatments to increase the biodegradability of PCBs generate alkaline wastewaters and alkalitrophic microorganisms would be ideal for the treatment of such waste streams. Studies which prove that an alkalitrophic PCB-degrading organism can grow and degrade PCBs at high pH are therefore very valuable. Alkaliphilic microorganisms have been readily isolated from a number of bacterial genera including *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Streptomyces* as well as from among eukaryotes, such as yeasts and fungi (Horikoshi, 1991). In particular, a large number of alkaliphilic bacilli have been intensively studied (Fukumori et al., 1989; Kaneko et al., 1989; Nakamura et al., 1991). However, there is no report about alkalitrophs or alkaliphiles which can degrade aromatic compounds such as PCBs/biphenyl,

and also no reports about alkalitrophs or alkaliphiles which belong to *Rhodococcus* sp. or *Dietzia* sp. (formerly *Rhodococcus maris*).

In order to gauge the breadth of microbial diversity, the small subunit (SSU) ribosomal RNA gene (16S rDNA) is a good marker as it has regions of both high conservation and very rapid evolution. It has also been sequenced in a vast array of organisms, which is useful in attempting to determine the phylogenetic history of the aromatic degraders.

In this paper, we isolated and characterized new alkalitrophic strains, including *Rhodococcus* sp., *Dietzia* sp. and *Alcaligenes* sp., which could degrade aromatic compounds such as biphenyl/PCBs. We also described the results of a phylogenetic analysis of the partial 16S SSU rRNA sequences.

A C-medium or an alkaline C-medium were used for screening. The C-medium consists of (per liter): (NH₄)₂SO₄, 5 g; KH₂PO₄, 2.93 g; K₂HPO₄, 5.87 g; MgSO₄ · 7H₂O, 0.3 g; NaCl, 2 g; CaCl₂, 0.03 g; FeSO₄ · 7H₂O, 0.01 g; NiSO₄ · 7H₂O, 0.6 mg; yeast extract, 0.2 g; and 2 ml of a trace elements solution. The trace elements solution contains 4 mg MoO₃, 28 mg ZnSO₄ · 5H₂O, 2 mg CuSO₄ · 5H₂O, 4 mg H₃BO₃, 4 mg MnSO₄ · 5H₂O, and 4 mg CoCl₂ · 6H₂O. Biphenyl was added to C-medium to a concentration of 0.5% or 0.1% as the carbon source. To adjust the pH of the medium, Na₂CO₃ or NaHCO₃ was added. For example, to adjust to pH 8.6, NaHCO₃ was added to the C-medium to a final concentration of 1%. To adjust to pH 10.0, Na₂CO₃ was added to the C-medium to a final concentration of 1%. Soil and water samples were collected in Arakawa River in Saitama, Heirinji and

* Address reprint requests to: Dr. Toshiaki Kudo, Laboratory of Microbiology, Institute of Physical and Chemical Research (RIKEN), 2–1 Hirosawa, Wako 351–0198, Japan.

Table 1. Properties of *Alcaligenes denitrificans* strain A41.

Criteria		Criteria	
Shape of cells	Rods	Hydrolysis of:	
Width (mm)	0.5–0.6	Starch	–
Length (mm)	1.0–2.5	Gelatin	–
		Casein	–
Motility	+	DNA	–
Flagella	Peritrichous	Tween 80	–
Gram reaction	–	Esculin	–
Lysis by 3% KOH	+		
Aminopeptidase (Cerny)	+	Tyrosine degradation	w
Spores	–		
		Utilization of:	
Oxidase	+	Acetate	+
		Adipate	+
Catalase	+	Caprate	+
		Citrate	+
Growth:		Malate	+
Anaerobic	–	Malonate	+
37/41°C	+/+	Phenylacetate	+
pH 5.6	+	L-Arabinose	–
Mac-Conkey-Agar	+	Glucose	–
SS-Agar	+	Mannose	–
Cetrimid-Agar	+	Maltose	–
		Mannitol	–
Pigments:		Gluconate	–
Non diffusible	–	2-Ketogluconate	+
Diffusible	–	N-Acetylglucosamine	–
Fluorescent	–	L-Serine	+
Pyocyanine	–	D-Xylose	–
		D-Galactose	–
Acid from (OF-Test):		Sucrose	+
Glucose aerobic	–	Pimelate	+
Glucose anaerobic	–	Sebacate	+
		Suberate	+
Gas from glucose	–	m-Tartrate	+
		Itaconate	+
Acid from (ASS):		Adonitol	–
Glucose	–	Sorbitol	w
Fructose	–	α -Ketoglutarate	+
Xylose	–	Azelate	+
		D-Malate	+
ONPG	–	3-Hydroxybenzoate	–
ADH	+	4-Hydroxybenzoate	–
Indole	+	Butylamine	–
VP	–		
Denitrification	+	NO ₂ from NO ₃	+
Levan from sucrose	–	Phenylalanine deaminase-	
Urease	+	lecithinase	–

Table 2. Characteristics of *Rhodococcus* sp. strain HA99.

Characteristics	HA99
Morphogenetic sequence	EB-R-C ^a
Color type of colony	Rose to beige
Cell wall type	meso-DAP ^b
Fatty acids	Tuberculostearic acid
Menaquinones	MK-8 (H ₂)
Mycolic acids (number of carbons)	38–46

^a Elementary branching-rod-coccus growth cycle.^b Diaminopimelic acid.Table 3. Characteristics of *Rhodococcus* sp. strain K37.

Characteristics	K37
Morphogenetic sequence	EB-R-C ^a
Color type of colony	Rose to beige
Cell wall type	meso-DAP ^b
Fatty acids	Tuberculostearic acid
Menaquinones	MK-8 (H ₂)
Mycolic acids (number of carbons)	30–50
Utilization of:	
N-Acetyl D-glucosamine	–
D-Glucosamine	–
D-Turanose	–
Citrate	+
Succinate	+
L-Leucine	–
Putrescine	–
3-Hydroxybenzoate	+
PNP- β -D-xyloside	+
D-Galactose	–
L-Rhamnose	–
Caprate	+
2-Hydroxyvalerate	–
L-Alanine	–
L-Proline	–
Tyramine	–
4-Hydroxybenzoate	+
PNP-phosphatidylcholine	–
D-Glucarate	–
D-Ribose	–
Arabitol	+
2-Oxoglutarate	–
4-Aminobutyrate	–
L-Serine	–
Acetamide	–
Phenylacetate	+
2-Deoxythymidine-s-PNP-phosphate-gluconate	–
Sucrose	–
Inositol	–
Pimelate	+
L-Aspartate	–
L-Valine	–
Quinate	+
Benzoate	+

^a Elementary branching-rod-coccus growth cycle.^b Diaminopimelic acid.

Kasumigaura. Alkalitrophic PCB/biphenyl-degrading bacteria were enriched from soil and water samples using the screening method described above. After 1-week cultivation, 0.2 ml of culture was spread onto alkaline plates (pH 9.0) for isolation. Colonies showing high 2,3-dihydroxybiphenyl dioxygenase (2,3-DHBD) activity were isolated and designated as strain A41, strain HA99 and strain K37.

Strains identified by means of their physiological, morphological and metabolic characteristics are shown in Tables 1–3. Strain A41 is an aerobic Gram-negative rod (0.5–0.6 by 1.0–2.5 mm), motile by peritrichous flagella, which is catalase and oxidase-posi-

tive, and for which the Oxidation-Fermentation-Test was negative. Strain A41 was identified as *Alcaligenes denitrificans* (Table 1). Strain HA99 and strain K37 were characterized as *Rhodococcus* sp. (Table 2) and *Rhodococcus* sp. (Table 3), respectively.

Strain TA1 was isolated as an alkalitrophic bacterium from an alkaline enrichment culture (pH 9.0) containing a termite sample. Four chemotaxonomic analyses have been carried out revealing the following results: diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Mycolic acid with a chain length of C₃₄–C₃₈ was detected. Fatty acid pattern is unbranched, saturated and unsaturated fatty acids plus a high amount of tuberculostearic acid. This pattern is typical for rhodococci and their relatives. Determination of the acyl-type of muramic acid is acetyl-type. All chemical markers found in strain TA1 correspond to the genus *Dietzia*, a taxon proposed by Rainey et al. (1995). However, as mentioned later, based on the partial 16S rDNA sequence, strain TA1 belongs to *Dietzia* but seems to be a species different from *Dietzia maris*.

The majority of PCB-degrading isolates characterized to date are Gram-negative bacteria within either the *Pseudomonas*, *Alcaligenes* or *Acinetobacter* genera. Information concerning the isolation and characterization of Gram-positive bacteria involved in the biodegradation of polychlorinated biphenyl compounds is quite sparse. Isolated strains were identified as *Alcaligenes denitrificans* and *Rhodococcus* sp. Alkalitrophic *Dietzia* sp. strain TA1 was isolated from termite ecosystem and found to degrade benzoate but not biphenyl. These results suggested that the population of *Rhodococcus* or relative species was more popular than Gram-negative bacteria among alkalitrophic, aromatic compound-degrading bacteria. However, the habitat and role of these alkalitrophic bacteria in the natural environment are not clear.

To measure the effect of pH on growth, strain A41 was cultured over a range of pH values in C-medium containing 0.1% (w/v) biphenyl, 4-chlorobiphenyl, benzoate or succinate at 30°C. A41 grew at pH 7–8, and at pH 9 after a short lag time, but no growth was observed at pH 10. When growth rate at various pHs was compared, growth rate was pH 7, 8 > pH 9 > pH 6 > pH 5 (no growth). Strain A41 grew at pH 10 after a long lag time in C-medium containing benzoate. Maximum enzyme activities were observed after 70–100 h cultivation in C-medium containing biphenyl at pH 8 or pH 9. However, when 4-chlorobiphenyl was the carbon source, cell growth and maximum enzyme activities were not as high as when biphenyl was the carbon source. When benzoate or succinate was used as the carbon source, maximum enzyme activities were very low. These results suggest that production of the

2,3-DHBD enzyme is induced by the addition of biphenyl effectively.

The effect of pH on growth for strain HA99 was measured. Bacteria were cultivated in C-medium containing 0.1% (w/v) biphenyl at 30°C at various pH values. HA99 grew at pH 7 and pH 8, and grew at pH 9 after a short lag time, while no growth was observed at pH 10. Maximum enzyme activities were observed after about 75 h cultivation in C-medium containing biphenyl at pH 8 and enzyme activities decreased quickly. To determine the effect of carbon sources for growth, HA99 was grown in C-medium (not containing yeast extract) in the presence of various carbon sources at pH 8. HA99 grew well in the presence of biphenyl and glucose as carbon sources, and maximum enzyme activity was observed after about 60 h cultivation. HA99 and K37 grew well in the presence of biphenyl, and no growth was observed in the absence of biphenyl. Maximum enzyme activities were observed after about 5 days of cultivation for strain K37 and after 10 days of cultivation for strain HA99, both in the presence of biphenyl.

None of them could grow at pH 10, suggesting a low frequency of alkaliphilic microorganisms which can degrade biphenyl/PCB in neutral soil samples. It is coincident with the fact that the frequency of alkaliphilic (including alkalitrophic) microorganisms in neutral soil samples is 10²–10⁵/g of soil, which corresponds to 10^{–2}–10^{–3} of the population of neutrophilic microorganisms.

To determine which PCB/biphenyl-related compounds could be used as a sole carbon source for growth, strains A41, HA99 and K37 were grown in C-medium containing various aromatic compounds. *Alcaligenes denitrificans* strain A41 utilized the following compounds as the carbon source for growth: biphenyl, 4-chlorobiphenyl and benzoate. Both *Rhodococcus* sp. HA99 and *Rhodococcus* sp. K37 utilized the following compounds for growth: biphenyl, 4-chlorobiphenyl, benzoate, 3-hydroxybenzoate, phenol and naphthalene. Furthermore, *Rhodococcus* sp. HA99 utilized protocatechuate and *Rhodococcus* sp. K37 utilized 4-hydroxybenzoate and catechol. *Dietzia* sp. strain TA1 utilized benzoate as the carbon source for growth but not biphenyl or 4-chlorobiphenyl.

We isolated co-existing bacteria from an enrichment culture of alkalitrophic PCB-degrading bacteria (unpublished data). These bacteria grew at an alkaline pH and were found to be co-existing bacteria which could degrade benzoic acid but not biphenyl nor polychlorinated biphenyls (PCBs). Co-culturing experiments suggested that the PCB/biphenyl-degrading bacteria K37 and HA99 provide benzoate for the growth of co-existing bacteria which were able to degrade benzoate. These results suggest that bacterial

Table 4. Degradation profile of PCBs assayed by KC300.

PCB structure	Degradation (%)		
	K37	HA99	A41
2-4, 2,3	100	100	100
4-4	100	100	83
2,5-2	100	100	74
2,4-2	93	100	93
2,6-3, 2,3,6	39	0	100
2,3-2, 2,6-4	41	55	88
2,5-3	100	100	97
2,4-3	100	100	100
2,5-4	84	94	97
2,4-4	88	95	100
3,4-2, 2,3,4, 2,3-3	97	97	100
2,3-4	48	68	100
3,4-4	52	64	88
2,5-2,6	5	14	51
2,3,6-2	0	14	38
2,3-2,6	1	14	34
2,5-2,5, 2,6-3,5	14	21	35
2,4-2,5	11	19	38
2,4-2,4, 2,4,5-2, 2,4,6-2	43	56	39
2,3-2,5	73	83	34
2,3-2,4, 2,3,6-3	33	44	55
2,6-3,4, 2,3,4-2, 2,3,6-4, 2,5-3,5	18	18	37
2,3-2,3	22	19	84
2,4,5-4	42	56	52
2,5-3,4, 3,4,5-2	39	51	47
2,4-3,4	38	50	71
2,3-3,4, 2,3,4-4	22	40	79

interactions for aromatic compound degradation existed in their natural habitats.

PCB degradation by these strains was measured by the method of Quensen et al. (1990). The PCB mixture used for PCB degradation analysis was KC300 (equivalent to Aroclor 1242), which contains mainly tri- or tetrachlorine substituted biphenyls. Some of the congeners were not identified because of the same retention times. Forty-one congeners were identified and quantified. From the degradation patterns (Table 4), PCB congeners substituted at position 2,6- were not degradable for either K37 or HA99 strains. PCB congeners which had either di- or trichlorine substituted biphenyls were degraded well by these strains (except for the PCB congeners 2,6-3 and 2,3,6, which were not degraded well by strain HA99). Congeners which had tetrachlorine substituted biphenyls were more recalcitrant for these strains.

The phylogenetic analysis of the 16S rDNA sequence was accomplished by PCR amplification of a 900 bp region within the approximately 1,500 bp rDNA gene using universal forward and reverse primers. The PCR primers contained 5' restriction-site-linkers and corresponded to nucleotide positions 519 to 533 of *E. coli* 16S rRNA for the forward primer (519FB; 5'-

ATTGGATCCCAGCMGCCGCGGTAA-3') and to *E. coli* position 1392 to 1405 for the reverse primer (1392RS; 5'-TGAGTCGACACGGGCGGTGTGTRC-3'), where R represents A or G and M represents A or C, with restriction site (underlined)-containing linkers at the 5' ends. The resulting PCR segments from each respective primer were then cloned and sequenced. The sequences were optimally aligned and phylogenies were constructed using the distance algorithms (Kimura, 1980) available in the PHYLIP suite of phylogenetic analysis programs. A phylogenetic tree was constructed from the distance by the neighbor-joining method (Saitou and Nei, 1987). The phenotypic characterization of the strains is in good agreement with the placement of the strains in the phylogenetic tree based on 16S SSU rRNA sequences. Strain A41 (D87096) was well identified as *Alcaligenes denitrificans*, a member of the β -subdivision of *Proteobacteria*. As shown in Fig. 1, strains HA99, K37 and TA1, which belong to Gram-positive bacteria with high G+C contents and are capable of aromatic compound degradation, form a cohesive clade which is in very good agreement with recent reports of rhodococci phylogeny. Strains HA99, K37 and TA1 were different species compared with the *Rhodococcus globerulus* strain P6, *Rhodococcus erythropolis* TA421 and *Rhodococcus* sp. strain RHA1 reported previously.

In phylogenetic analysis, the question of primary interest is the origin: how did these particular organisms obtain (i.e. by descent or by recombination) their particular aromatic compound degradation pathways? If the genes within the pathways are more similar than our neutral markers or present significantly different patterns of inheritance, then we can presume that the genes may be moving from background to background (and species to species) independent of their direct descent from parent to offspring. The small subunit ribosomal RNA gene (16S rDNA) is a good marker for this test as it has regions of both high conservation and very rapid evolution. Recently, several *Rhodococcus* sp. strains such as *Rhodococcus globerulus* strain P6 (Asturias and Timmis, 1993), *Rhodococcus erythropolis* TA421 (Chung et al., 1994; Maeda et al., 1995) and *Rhodococcus* sp. strain RHA1 (Masai et al., 1995) were isolated and characterized genetically. These results strongly suggest that the *Rhodococcus* group is one of the important groups for the degradation of aromatic compounds and that *Rhodococcus* sp. strain K37, *Rhodococcus* sp. strain HA99 and *Dietzia* sp. strain TA1 are new species which can degrade biphenyl/PCBs or aromatic compounds.

It was reported that multiple *bphC* genes were observed in *Rhodococcus* sp. Southern hybridization experiments suggest that *Rhodococcus* sp. strain K37

Fig. 1. Phylogenetic analysis of high G+C subdivision: *Nocardia* and mycobacteria assemblage.

Aromatic-degrading strains and small subunit (SSU) rDNA sequences reported here are shown in boxes. Aromatic-degrading strains are shown in bold. The sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers mentioned in the figure. The scale bar represents the average number of substitutions per position.

and *Rhodococcus* sp. strain HA99 have several DNA bands which hybridized with *bphC* genes from *Rhodococcus erythropolis* TA421 (unpublished data). Therefore, an analysis of the genes responsible for PCBs/biphenyl or aromatic compound degradation from alkalitrophic bacteria will give us a broad perspective on the evolution of catabolic functions. Genetic analysis of the genes responsible for PCBs/biphenyl degradation from alkalitrophic bacteria is now in progress.

In conclusion, we isolated and characterized new

alkalitrophic strains, including *Rhodococcus* sp., *Dietzia* sp. and *Alcaligenes* sp., which could degrade aromatic compounds such as biphenyl/PCBs. We also described the results of a phylogenetic analysis of the partial 16S SSU rRNA sequences.

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