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Evaluation of Strains Isolated by Growth on Naphthalene and Biphenyl for Hybridization of Genes to Dioxygenase Probes and Polychlorinated Biphenyl-Degrading Ability

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Approximately equal numbers of bacteria were isolated from primarily tropical soils by growth on biphenyl and naphthalene to compare their competence in polychlorinated biphenyl (PCB) degradation. The strains isolated by growth on biphenyl catalyzed more extensive PCB degradation than the strains isolated by growth on naphthalene, suggesting that naphthalene cocontamination may be only partially effective in stimulating the cometabolism of lower chlorinated PCBs. Probes were made from the *bph*, *nah*, and *tod* genes encoding the large iron sulfur protein of the dioxygenase complex and hybridized to 19 different strains. The hybridization patterns did not correlate well with the substrates of isolation, suggesting that there is considerable diversity in these genes in nature and that probe hybridization is not a reliable indication of catabolic capacity. The strains with the most extensive PCB degradation capacity did strongly hybridize to the *bph* probe, but a few strains that exhibited strong hybridization had poor PCB-degrading ability. Of the 19 strains studied, 5 hybridized to more than one probe and 2, including one strong PCB degrader, hybridized to all three probes. Southern blots showed that the *bph* and *nah* probes hybridized to separate bands, suggesting that multiple dioxygenases were present. Multiple dioxygenases may be an important feature of competitive decomposers in nature and hence may not be rare. Most of the isolates identified were members of the beta subgroup of the *Proteobacteria*, a few were gram positive, and none were true *Pseudomonas* species.

Polychlorinated biphenyls (PCBs) are pollutants that are widely distributed in the environment. In 1973 Ahmed and Focht described two bacterial species that grow on biphenyl and 4-chlorobiphenyl (1). Since then, the naturally occurring bacteria that are capable of cooxidizing PCBs, the degradation pathway, and the genes that encode the biphenyl-oxidizing pathway have been studied extensively (8, 10, 13).

The dioxygenases responsible for the first step in the aerobic oxidation of aromatic hydrocarbons such as naphthalene, biphenyl, benzene, and certain other aromatic compounds have many similarities, suggesting that they have a common if distant evolutionary origin (10, 26). The aerobic metabolism of PCBs is dependent on enzymes of this class, although the abilities of particular enzymes and hence strains to metabolize various congeners of PCBs vary widely (4, 13). Since PCB degraders cometabolize PCBs and do not grow on the PCB congeners found in nature, a growth substrate that selects for a competent PCB-degrading population is needed. Biphenyl has been used as the carbon source for stimulating PCB-degradative activity, but it is expensive and often subject to regulatory restrictions as well. Hence, other carbon sources that enrich PCB-cometabolizing populations are needed. Kuhm et al. (18) demonstrated that most enzymes synthesized by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1 can function both in the naphthalene-degradative pathway and in the biphenyl-degradative pathway. Hence, naphthalene could be a growth substrate for PCB cometabolizers if such strains were sufficiently active on PCBs. Naphthalene and other aromatic hydrocarbons occur as cocontaminants with PCBs at some sites, and naphthalene is found at low concentrations in most natural soils (16).

We isolated similar numbers of bacteria by growth on biphenyl and on naphthalene from soil and sediments from several sites and evaluated their abilities to degrade PCBs. We also used *nah*, *bph*, and *tod* probes to screen for the diversity of dioxygenase genes in the isolates and evaluated whether these probes were useful in predicting PCB biodegradability.

MATERIALS AND METHODS

Samples. Samples from Brazil, Puerto Rico, and New York were used in this study (Table 1). Cubatão, Brazil, which is 55 km from São Paulo, is the site of many petrochemical and metalurgical industries, which discharge more than 3×10^6 tons (ca. 2.7×10^6 metric tons) of industrial wastes per year. Jabaquara is a neighborhood in the city of Cubatão. No PCBs were detected in any of the Brazilian samples.

Enrichment cultures and isolation. Enrichment cultures were prepared in parallel by using 0.05% (wt/vol) naphthalene and 0.05% (wt/vol) biphenyl as carbon sources in a mineral medium (11). The cultures were shaken at 30°C until good growth was observed (approximately 4 days). After three serial transfers in the enrichment medium, the cultures were streaked onto a solid mineral medium in a petri dish which had biphenyl or naphthalene in the lid. All colonies that differed in appearance were picked and restreaked two additional times. Isolates were then streaked onto tryptic soy agar to check for purity.

Characterization of isolates. Isolates were characterized for similarity and grouped by using data from gram-negative BIOLOG microplates and chromosomal band patterns obtained by PCR performed with repetitive extragenic palindromic (REP) primers. The BIOLOG procedures used were the procedures described in the manufacturer's manual, and a similarity tree was calculated by using NTSYS-pc software (version 1.7). REP-PCR patterns were determined for all 48 isolates studied as described by de Bruijn (6), except that template DNA was extracted from each strain by using the standard proteinase K-hexadecyltrimethylammonium bromide procedure (3). The same batch of primers was used for all strain comparisons. A fatty acid methyl ester analysis was conducted

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TABLE 1. Sites and numbers of isolates obtained from biphenyl or naphthalene enrichment cultures

Site	Description	Substrate used for enrichment	No. of isolates
Pinheiros River, Brazil	Sediment contaminated with industrial effluents	Biphenyl	11
		Naphthalene	14
Jabaquara, Brazil	Soil from area adjacent to a PCB-contaminated site	Biphenyl	5
		Naphthalene	6
Cubatão, Brazil	Soil from a landfill contaminated with chlorohydrocarbons	Biphenyl	2
		Naphthalene	3
San Juan, Puerto Rico	Soil contaminated with PCBs	Biphenyl	3
		Naphthalene	3
Glenn Falls, N.Y.	Drag strip soil containing PCB-contaminated oils	Biphenyl	1

as described in the MIDI manual (Microbial Identification, Inc., Newark, Del.). Three replicate cultures were analyzed for each isolate.

The isolates were tested on agar plates for the ability to grow on biphenyl and on naphthalene by the protocol that was used for the original isolation.

Assay for the degradation of PCB congeners. Isolates were screened as resting cells for PCB-degrading ability by using the protocol described by Bedard et al. (4), except that the cells were grown on the substrate of isolation, either biphenyl or naphthalene. Each PCB congener was used at a final concentration of 2 ppm. 2,4,6,2′,4′,6-Chlorobiphenyl was used as the internal standard. Each assay was performed in duplicate. A control consisting of heat-inactivated cells was run in parallel for each strain.

After 24 h of incubation at 30°C, the flasks were extracted three times by using 4 volumes of hexane. The final volume was adjusted to 1 ml before analysis. Samples were analyzed by using a gas chromatograph (model 5890; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a Hewlett-Packard Ultrabond 1 capillary column (type SE-54 equivalent; 50 m by 0.2 mm) and an electron capture detector, as described by Quensen et al. (20).

Dioxygenase probes. The probes used were DNA fragments from the initial region of the genes coding for the large iron sulfur protein of the dioxygenase complex of *bph* (*bphA1* of *Pseudomonas pseudoalcaligenes* K707), *nah* [*nahA3* of *Pseudomonas putida* BS202(NPL1)], and *tod* (*todC1* of *P. putida* F1). The *bph* gene was provided by Kensuke Furukawa, the *nah* gene was provided by Alexander Boronin, and the *tod* gene was provided by David Gibson. The level of similarity between the *nahA3* probe and the similar region of well-studied plasmid NAH 7 was 94%. The levels of similarity of *tod* to *nah*, *nah* to *bph*, and *bph* to *tod* are 57, 58, and 71%, respectively, for the region from which the probes were constructed. The DNA fragments used were the 0.47-kb *Sac*I-*Mlu*I fragment of pKTF 18 (23) for *bph*, the 0.55-kb *Sma*I-*Kpn*I fragment of pBS 959 for *nah* (5), and the 0.72-kb *Eco*RI-*Stu*I fragment of pDTG 601 (27) for *tod*. A 0.85-kb probe from *nahC* coding for 1,2-dihydroxynaphthalene dioxygenase in *P. putida* BS202 was obtained from the *Sac*I-*Nco*I fragment of pBS 955 (25). The probes were labeled with ^{32}P by using a nick translation kit (Boehringer Mannheim, Indianapolis, Ind.) and were purified with a Geneclean kit (Bio 101, Inc., La Jolla, Calif.).

DNA blot preparation and probe hybridization. Filters for colony blot, slot blot, and Southern blot analyses were prepared by standard methods (19). Three identical slot blot membranes were prepared for hybridization with the three dioxygenase probes. Genomic DNA (1.0 μg) from each isolate and known dilutions of the plasmids from which the probes were obtained were spotted onto each filter. For Southern blots, 2 μg of DNA was digested with *Xho*I and *Bam*HI. Product separation and transfer were performed as described by Maniatis et al. (19). DNA was fixed to the membranes by UV cross-linking (Stratagene, La Jolla, Calif.). Prehybridization and hybridization were performed by standard methods (19). High-stringency conditions were used to wash the blots; the first wash was at 30°C, the second wash was at 50°C in 0.5× SSC–0.1% sodium dodecyl sulfate (SDS) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the third wash was at 68°C in 0.1× SSC–0.1% SDS (19).

RESULTS

Isolate grouping. A total of 48 isolates were selected from the initial enrichment cultures (Table 1); 26 of these organisms were isolated by growth on naphthalene, and 22 were isolated by growth on biphenyl. When these isolates were clustered on the basis of BIOLOG substrate use patterns, two major clusters (groups I and II) were apparent (Fig. 1). Group I contained 25 isolates, predominantly from naphthalene enrichment cultures; only 5 of these isolates were from biphenyl enrichment cultures. Group II contained 16 isolates, mainly from biphenyl enrichment cultures; only 2 of the group II isolates were from naphthalene enrichment cultures. Group I was subdivided into four clusters which correlated with the origins of the isolates. All 17 subgroup IA isolates were obtained from the Pinheiros River sediment, and subgroup IB contained 5 isolates obtained from Jabaquara. Subgroups IC and ID contained only three isolates. Seven isolates could not be evaluated by BIOLOG, usually because they did not grow on the standard medium.

A total of 19 different REP-PCR patterns were obtained for the 48 isolates tested. The strains differentiated by the REP-PCR results correspond reasonably well to the 13 different clusters differentiated by the BIOLOG analysis (Table 2). The isolates clustered in subgroup IA by the BIOLOG analysis produced the same REP-PCR pattern (cluster 3); this subgroup contained primarily naphthalene-enriched strains, but it also contained five isolates obtained from biphenyl enrichment cultures. The single subgroup IC isolate, strain 33N, produced the same REP pattern as the subgroup IA isolates. Four different REP patterns were produced by the 25 Pinheiros River isolates. Most of the Jabaquara isolates that clustered in subgroup IB on the basis of the results of the BIOLOG analysis produced the same REP pattern; the only exception was isolate

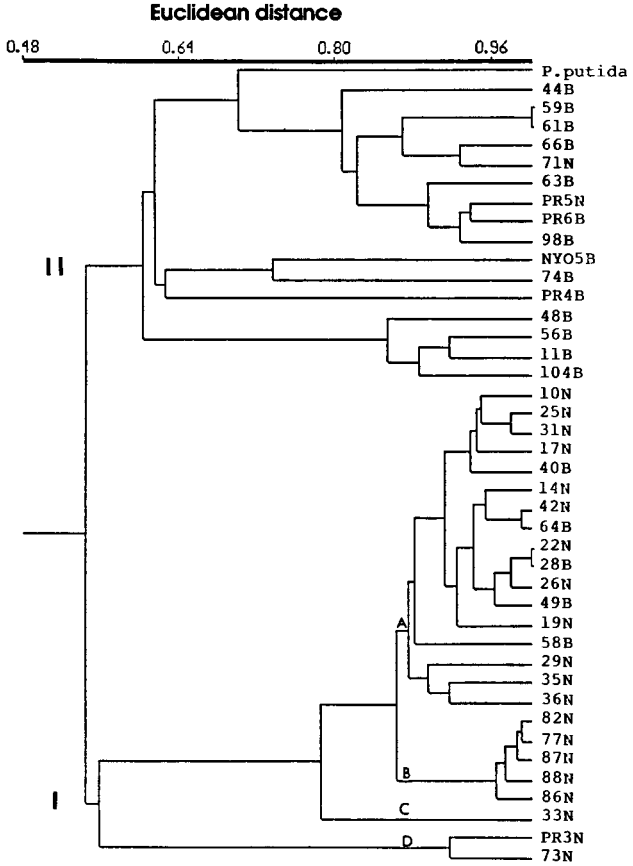

FIG. 1. Dendrogram for 42 isolates derived from the BIOLOG analysis. The clusters (groups I and II), strain designations, and substrates of isolation (biphenyl [B] or naphthalene [N]) are indicated.

TABLE 2. Characteristics of selected isolates representing each REP pattern that were isolated by growth on biphenyl or naphthalene

Site	Strain	Cluster as determined by:		Substrate of isolation[a]	Growth on[b]:		Hybridization to[c]:			PCB-degradative competence[d]	Species as determined by fatty acid methyl ester analysis[e]	Similarity index[f]
		BIOLOG analysis	REP-PCR analysis		Biphenyl	Naphthalene	*bph* probe	*nah* probe	*tod* probe			
Pinheiros River	44	1	1	B	+	+	+++	+++	+	+++	*Comamonas testosteroni*	0.41
Pinheiros River	48	2	2	B	+	−	−	−	−	+	*Xanthomonas maltophilia*	0.64
Pinheiros River	49	3	3	B	+	+	++++	+	+	+	*Pseudomonas gladioli*	0.60
Pinheiros River	63	4	4	B	+	−	++++	+	+	++++		
Jabaquara	86	5	5	N	−	+	−	++++	−	+	*Pseudomonas gladioli*	0.71
Jabaquara	87	5	6	N	−	+	−	++++	−	+	*Pseudomonas gladioli*	0.66
Jabaquara	90	ND[g]	7	N	−	+	−	−	−	++	*Xanthomonas maltophilia*	0.66
Jabaquara	98	6	8	B	+	−	++	−	−	−	*Alcaligenes xylosoxidans*	0.76
Jabaquara	103	ND	9	B	+	−	−	−	−	ND		
Cubatão	11	7	10	B	+	−	−	−	−	++	*Xanthomonas maltophilia*	0.50
Cubatão	69	ND	11	N	−	+	++	−	−	ND	NG	
Cubatão	71	8	12	N	−	+	−	−	−	−	*Pseudomonas gladioli*	0.21
Cubatão	73[h]	9	13	N	−	+	+++	−	−	−	*Staphylococcus warnerii*	0.81
New York	NY05[h]	10	14	B	+	−	++	−	+	++++	*Rhodococcus erythropolis*	0.26
Puerto Rico	PR1[h]	ND	15	B	±	−	+	++++	−	−	*Micrococcus luteus*	0.71
Puerto Rico	PR2	ND	16	N	−	−	+	−	−	ND		
Puerto Rico	PR3	11	17	N	−	+	−	−	−	−		
Puerto Rico	PR4	12	18	B	+	−	−	−	−	−		
Puerto Rico	PR5	13	19	N	−	±	++	−	−	ND	*Alcaligenes xylosoxidans*	0.49

[a] B, biphenyl; N, naphthalene.
[b] +, growth; −, no growth; ± poor growth.
[c] −, no hybridization; + to ++++, hybridization, with the strength of the hybridization signal increasing from + to ++++.
[d] −, <20% loss of PCB congener(s); +, >20% loss of dichlorobiphenyl congeners; ++, >20% loss of di- and trichlorobiphenyl congeners; +++, >20% loss of di-, tri-, and tetrachlorobiphenyl congeners; ++++, >20% loss of di-, tri-, tetra-, and pentachlorobiphenyl congeners.
[e] NG, no growth occurred on fatty acid methyl ester test medium. No taxon is given if the similarity index was <0.5 and it was not consistent with the staining properties, morphology, or habitat.
[f] Means of values obtained from three analyses.
[g] ND, not determined.
[h] These strains were gram-positive cocci; all other strains were gram-negative rods.

87, which produced a REP pattern very similar to the patterns of the other isolates obtained from Jabaquara (strains 82, 77, 88, 86). The isolates obtained from Cubatão and Puerto Rico produced four and five different patterns, respectively. The isolate obtained from New York produced an additional distinct pattern.

PCB degradation. A total of 20 of the 48 strains were evaluated for the ability to degrade PCB congeners. The results obtained for representative isolates are shown in Table 3, and other results are summarized in Table 2. Percentages of congener removal greater than 20% were considered significant biodegradation. The competence for PCB degradation varied considerably among the isolates. Strains 44, NY05, and 63 (data not shown) exhibited the most extensive PCB degradation and were placed in the category of greatest competence. Strains NY05 and 63 degraded significant amounts of pentachlorobiphenyl congeners. Strains 29, 35, and 49 produced the same REP pattern but differed significantly in the congeners which they metabolized. None of the isolates obtained from highly contaminated Puerto Rican soils exhibited significant PCB degradation (Table 2).

Hybridization. All naphthalene and biphenyl isolates were screened by performing colony hybridization with probes for the naphthalene upper pathway (*nahA3*) and lower pathway (*nahC*). Of the 26 naphthalene-selected strains studied, 19 (73%) hybridized to the *nahA3* and *nahC* probes, and only 6 of 25 (24%) of the biphenyl-selected strains studied hybridized to these probes (Table 4). All 25 strains that hybridized to one *nah* probe also hybridized to the other. This suggests that the *nah* upper and lower pathway sequences are probably conserved in these organisms.

One isolate that was representative of each REP-PCR pattern was evaluated for hybridization of its DNA to the dioxygenases probes (Fig. 2). The *bph* probe, which exhibits 71% similarity to the *tod* gene, showed some hybridization to the DNA of *P. putida* F1, the strain from which the *tod* gene was sequenced, but the *nah* probe, which exhibits only 57% similarity to the *tod* gene, showed no substantive hybridization to strain F1 (Fig. 2A and B, respectively). These results serve as a reference point for the hybridization detection limit. The hybridization results are compared with the substrate of isolation, growth substrates, and PCB degradation ability in Table 2. All but two isolates (strains 44 and 49) grew only on the substrate of isolation, and these strains grew on both naphthalene and biphenyl.

The three probes revealed a rather random pattern of hybridization relative to the substrate of isolation. Eight isolates (strains 44, 49, 63, 86, 87, 98, NY05, and PR1) hybridized to probes for the substrate of isolation, and nine isolates hybridized to one or two probes for substrates different from the substrates used for isolation. Three isolates (strains 44, 49, and 63) hybridized to all three probes. The strongest hybridization, however, occurred between isolates and the probe corresponding to the substrate of isolation (isolates 49, 63, 86, and 87), except for isolate PR1 on biphenyl, which exhibited a strong signal with the *nah* probe. The most frequent pattern was hybridization to no probes. Five strains hybridized to more than one probe.

TABLE 3. Competence for degrading PCB congeners of strains isolated by growth on naphthalene or biphenyl and strains that produce identical REP patterns[a]

PCB congener	% Removed by:						
	Strain 44 (B)	Strain 29[b] (N)	Strain 35[b] (N)	Strain 49[b] (B)	Strain 90 (N)	Strain 104 (B)	Strain NY05 (B)
2,3	72	56	23	97	75	49	99
2,2′	92	7	2	6	90	20	7
2,4′	100	70	9	98	45	57	99
2,5,2′	60	5	0	0	54	17	20
2,5,4′	55	4	0	1	29	13	54
2,3,2′,3′	67	0	0	0	0	2	92
2,3,2′,5′	29	0	0	0	0	3	64
2,5,3′,4′	26	0	9	1	4	3	24
2,5,2′,5′	25	0	0	0	0	4	7
2,4,5,2′,5′	7	0	0	2	1	8	4
2,3,4,2′,5′	8	0	6	4	0	0	68
2,4,5,2′,3′	12	0	0	0	4	0	80
4,4′	95	9	0	92	0	24	98
2,4,4′	95	8	0	4	0	5	97
2,4,3′,4′	53	0	0	0	2	4	85
2,4,2′,4′	15	0	4	1	15	0	2
3,4,3′,4′	36	0	0	0	5	10	22
2,4,5,2′,4′,5′	4	0	2	8	0	0	0

[a] Data are means of the values obtained in replicate analyses after comparison with an inactivated control. (B) and (N) indicate isolation on biphenyl and naphthalene, respectively.
[b] Strains 29, 35, and 49 produced identical REP patterns.

Strains 44 and 49, which hybridized to both the *bph* probe and the *nah* probe, were analyzed by Southern blotting to determine if hybridization occurred in the same restriction fragment. The bands hybridizing to *bph* and *nah* were in different positions in both restriction digests (Fig. 3), suggesting that these organisms may carry two separate dioxygenase genes. Two sets of isolates that produced the same REP patterns but were isolated with different substrates were also analyzed on Southern blots to see if they were the same strains. These isolates were strains 35 and 49 from the Pinheiros River and strains 73 and 104 from Jabaquara. Differences in hybridization and band position were observed for both pairs, which showed that they are different strains (Fig. 3).

Taxonomic characterization. The 19 isolates that produced different REP patterns and were analyzed by hybridization were partially characterized by performing Gram staining, morphological, and fatty acid methyl ester analyses (Table 2). The similarity indices were rather high for most strains. The strains may belong to the species indicated in Table 2 or to close relatives. One gram-positive isolate, strain NY05, grew unusually well and produced high yields on biphenyl and had an intense yellow pigment when biphenyl was the carbon source. Most of the strains related to *Pseudomonas* (*Burkholderia*) *gladioli* produced a brown color in liquid medium when naphthalene was the carbon source.

TABLE 4. Distribution of *nahA3*- and *nahC*-hybridizing colonies with respect to substrate of isolation and origin

Geographic origin	No. of strains that hybridized to the *nah* probes/no. of strains isolated on naphthalene	No. of strains that hybridized to the *nah* probes/no. of strains isolated on biphenyl
Pinheiros River	14/15	6/13
Jabaquara	5/6	0/7
Cubatão	0/2	0/2
Puerto Rico	0/3	0/3

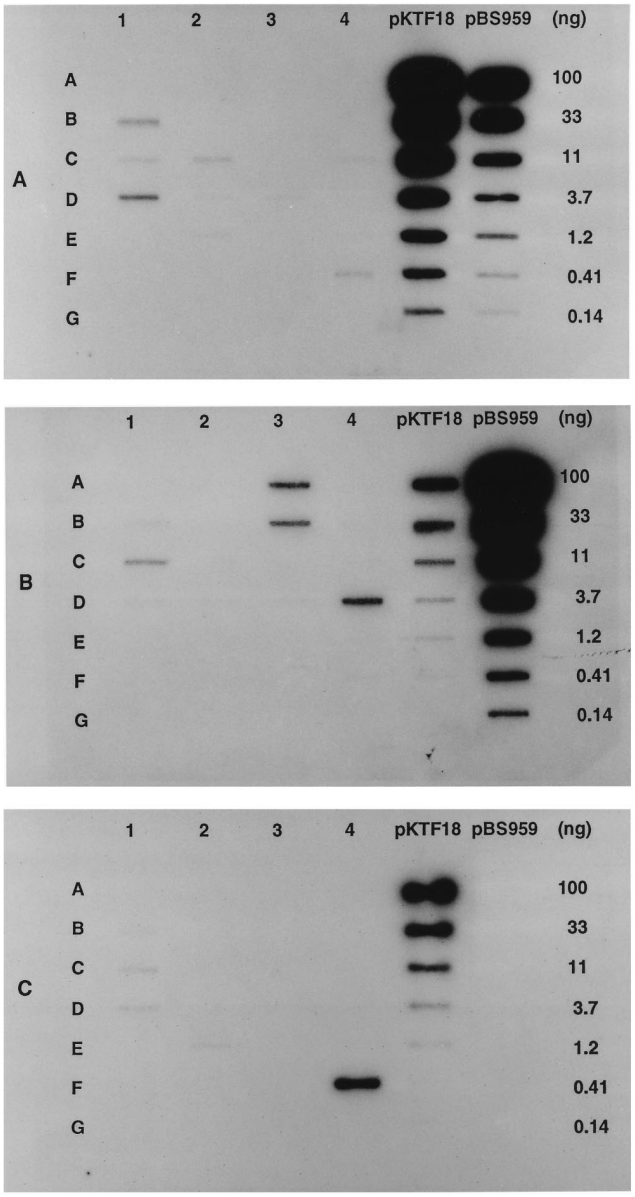

FIG. 2. Slot blot hybridization of the *bph* (A), *nah* (B), and *tod* (C) probes to selected isolates. Column 1, strains 48, 49, 44, and 63; column 2 strains 11, 71, 73, 69, and NY05; column 3, strains 86, 87, 90, 98, and 103; column 4, strains PR4, PR3, PR5, PR1, and PR2 and *P. putida* F1. Standard pKTF18 contained the *bph* gene, and standard pBS959 contained the *nah* gene.

DISCUSSION

The most PCB-competent strains isolated by growth on naphthalene did not degrade as many PCB congeners as the most competent strains isolated by growth on biphenyl degraded. However, some naphthalene-selected strains had weak or moderate PCB-degrading competence (rating, + to ++). This suggests that most populations selected by growth on

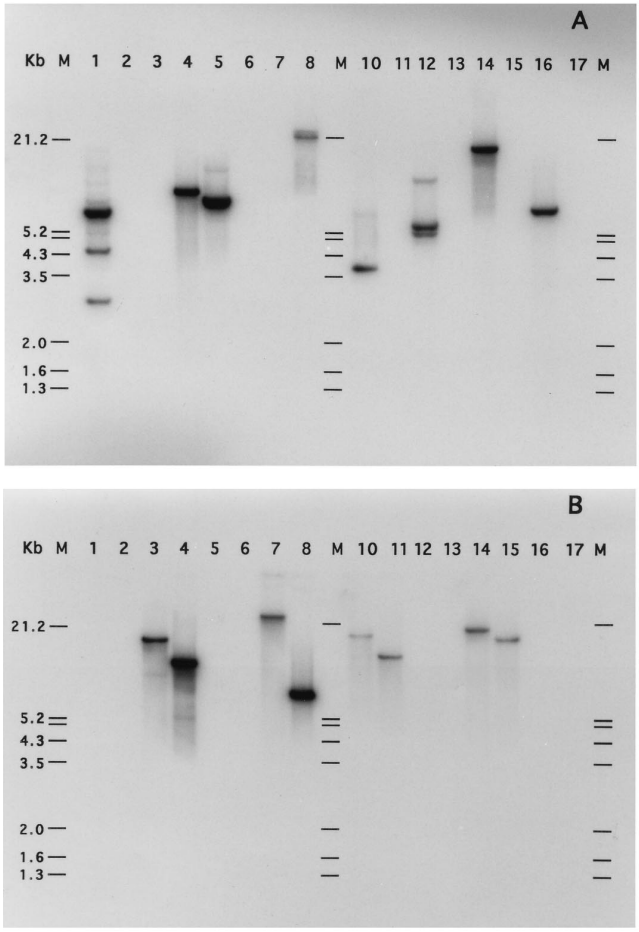

FIG. 3. Hybridization of a Southern blot to the *bph* (A) and *nah* (B) probes. Lanes M, marker; lanes 1 and 5, strain 63; lanes 2 and 6, strain NY05; lanes 3 and 7, strain 86; lanes 4 and 8, strain 44; lanes 10 and 14, strain 49; lanes 11 and 15, strain 35; lanes 12 and 16, strain 104; lanes 13 and 17, strain 73. Lanes 1 through 4 and 10 through 13 were digested with *Xho*I, and lanes 5 through 8 and 14 through 17 were digested with *Bam*HI. The positions of size markers (in kilobases) are indicated on the left.

naphthalene may have only limited PCB-metabolizing ability and that naphthalene (and perhaps other aromatic cocontaminants) may be only marginally effective in stimulating the cometabolism of PCBs. Perhaps this activity would be sufficient to stimulate degradation of the products of more extensive reductive dechlorination of Aroclors 1242 and 1248 (20). More encouraging results were recently obtained by Hernandez et al. (14), who found that natural aromatic compounds in plants stimulated complete degradation of Aroclor congeners.

The correlation between hybridization to dioxygenase probes and substrate of isolation was poor, suggesting that there is considerable diversity in the genes in nature and that such probes are not reliable indicators of catabolic capacity. However, the three strains with the highest levels of PCB-degrading ability did hybridize to the *bph* probe, but two strains that hybridized strongly to this probe exhibited only moderate to weak activity on PCBs. There appeared to be little correlation between *bph* hybridization and PCB degradation for the strains in the none-to-moderate (++) PCB degradation category. Similarly, the *nah* and *tod* hybridization results did not improve the predictability of PCB degradation capacity. This finding is similar to what appears to be the case for many catabolic traits, namely, that sequence divergence is rather extensive and hence probes made from gene fragments that are several hundred base pairs long are probably not reliable predictors of biodegradation potential. In the case of PCBs, it is now known that single-base-pair changes can affect which congeners are metabolized (7, 17). Eventually it may be possible to make more specifically designed primers or probes by using such information.

In two strains that hybridized to both the *bph* probe and the *nah* probe, two separate dioxygenases may be present since the hybridization bands were at different positions on Southern blots and the strains grew on both substrates, a rare characteristic among the isolates studied. If there are two genes, we do not know which was induced or active in PCB congener degradation. Strains with multiple aromatic dioxygenases might have enhanced catalytic ability. One of these strains, strain 44, was among the most effective PCB degraders. Since diversity in dioxygenases appears to be high, there may even be additional nonhybridizing genes that also contribute to PCB degradation in these strains. Multiple aromatic oxygenase genes have been found in other biphenyl-degrading strains (2, 22) and toluene-degrading strains (12, 15), suggesting that this may be a common feature of competitive decomposers in nature.

Analysis of new isolates in a collection with REP-PCR primers (or similar primers) has been an efficient method for eliminating siblings or closely related strains from a study (9, 24). In this study we observed important phenotypic diversity in strains belonging to the same REP group. Some of these differences were differences in the PCB congeners degraded, in the bands on Southern blots, in the substrates oxidized in BIOLOG plates, and in colony morphology. In general, the extent of the differences within the a REP group was not large; for example, all REP group 3 strains degraded PCB weakly, although some of the congeners degraded were different. The results of the BIOLOG analysis provided a rapid and useful way to group isolates.

The results of the taxonomic characterization which was performed suggested that members of the beta subgroup of the *Proteobacteria* were the most common isolates, and seven strains were similar to *Burkholderia gladioli*, *Alcaligenes xylosoxidans*, and *Comamonas testosteroni*; three strains were gram-positive organisms, and three strains were similar to *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia* and *Pseudomonas maltophilia*). It is surprising that no isolates were true *Pseudomonas* strains (members of the gamma subgroup of the *Proteobacteria*). It is known that some strains of *Pseudomonas stutzeri* grow on naphthalene and carry genes highly homologous to the *nah* genes on plasmid NAH 7 (21) and that the *bph* and *nah* probes used in this study came from *P. putida*, a species belonging to the true *Pseudomonas* group. Nonetheless, it is clear that the importance of *Pseudomonas* species in aromatic biodegradation in nature may have been overestimated now that better isolation methods and taxonomic information are available.

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REFERENCES

1. **Ahmed, M., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. **19:**47–52.
2. **Asturias, J. A., and K. N. Timmis.** 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. J. Bacteriol. **175:**4631–4640.
3. **Ausubel, M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Current protocols in molecular biology, vol. I. Greene Publishing Associates, New York.
4. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.
5. **Boronin A. M., T. V. Tsoi, I. A. Kosheleva, M. U. Arinbasarov, S. A. Selifonov, and A. G. Kozlovsky.** 1989. Cloning of the primary steps of naphthalene oxidation in *Escherichia coli* cells. Genetika **25:**226–237.
6. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprinting the genomes of *Rhizobium meliloti* and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
7. **Erickson, B. D., and F. J. Mondello.** 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858–3862.
8. **Focht, D. D.** 1995. Strategies for the improvement of aerobic metabolism of polychlorinated biphenyls. Curr. Opin. Biotechnol. **6:**341–346.
9. **Fulthorpe, R. R., C. McGowan, O. V. Maltseva, W. E. Holben, and J. M. Tiedje.** 1995. 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. **61:**3274–3281.
10. **Furukawa, K.** 1994. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation **5:**289–300.
11. **Furukawa, K., F. Matsumura, and K. Tonomura.** 1978. *Alcaligenes* and *Acinetobacter* strains capable of degrading polychlorinated biphenyls. Agric. Biol. Chem. **42:**543–548.
12. **Haigler, B. G., C. A. Pettigrew, and J. C. Spain.** 1992. Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **58:**2237–2244.
13. **Hernandez, B. S., J. J. Arensdorf, and D. D. Focht.** 1995. Catabolic characteristics of biphenyl-utilizing isolates which cometabolize PCBs. Biodegradation **6:**75–82.
14. **Hernandez, B. S., M. Chial, S.-C. Koh, and D. D. Focht.** 1995. Plant terpenes are the natural substrates for indigenous soil bacteria that utilize biphenyl, abstr. N130. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
15. **Johnson, G. R., and R. H. Olsen.** 1995. Nucleotide sequence analysis of genes encoding a toluene/benzene 2-monooxygenase from *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **61:**3336–3346.
16. **Jones, K. C., J. A. Stratford, K. S. Waterhouse, and N. B. Vogt.** 1989. Organic contaminants in Welsh soils: polynuclear aromatic hydrocarbons. Environ. Sci. Technol. **5:**540–550.
17. **Kimura, N., and K. Furukawa.** 1995. Construction of chimera biphenyl dioxygenases and functional analysis, p. 102. *In* Abstracts of *Pseudomonas* 1995: Fifth International Symposium on *Pseudomonas*, Tsukuba, Japan.
18. **Kuhm, A. E., A. Stoz, and H.-J. Knackmuss.** 1991. Metabolism of naphthalene by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1. Biodegradation **2:**115–120.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring, Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Quensen, J. F., III, J. M. Tiedje, and S. A. Boyd.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360–2369.
21. **Rossello-Mora, R. A., J. Lalucat, and G. Garcia-Caldes.** 1994. Comparative biochemical and genetic analysis of naphthalene degradation among *Pseudomonas stutzeri* strains. Appl. Environ. Microbiol. **60:**966–972.
22. **Seto, M., K. Kimbara, M. Shimura, T. Hatta, M. Fukuda, and K. Yano.** 1995. A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **61:**3353–3358.
23. **Taira, K., J. Hirose, J. Hayashida, and F. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.
24. **Tonso, N. L., V. G. Matheson, and W. E. Holben.** 1995. Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D. Microb. Ecol. **30:**1–2.
25. **Tsoi, T. V., I. A. Kosheleva, V. S. Zamaraev, S. A. Selifonov, O. V. Trelina, I. I. Starovoitov, and A. M. Boronin.** 1988. Cloning and expression of *Pseudomonas putida* genes controlling catechol-2,3-oxygenase activity in *Escherichia coli* cells. Genetika **24:**1550–1561.
26. **Williams, P. A., and J. R. Sayers.** 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation **5:**195–217.
27. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1. J. Biol. Chem. **264:**14940–14946.