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Molecular Relationship of Chromosomal Genes Encoding Biphenyl/Polychlorinated Biphenyl Catabolism: Some Soil Bacteria Possess a Highly Conserved *bph* Operon

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All the genes we examined that encoded biphenyl/polychlorinated biphenyl (PCB) degradation were chromosomal, unlike many other degradation-encoding genes, which are plasmid borne. The molecular relationship of genes coding for biphenyl/PCB catabolism in various biphenyl/PCB-degrading *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Moraxella*, and *Arthrobacter* strains was investigated. Among 15 strains tested, 5 *Pseudomonas* strains and one *Alcaligenes* strain possessed the *bphABC* gene cluster on the *Xho*I 7.2-kilobase fragment corresponding to that of *Pseudomonas pseudoalcaligenes* KF707. More importantly, the restriction profiles of these *Xho*I 7.2-kilobase fragments containing *bphABC* genes were very similar, if not identical, despite the dissimilarity of the flanking chromosomal regions. Three other strains also possessed *bphABC* genes homologous with those of KF707, and five other strains showed weak or no significant genetic homology with *bphABC* of KF707. The immunological cross-reactivity of 2,3-dihydroxybiphenyl dioxygenases from various strains corresponded well to the DNA homology. On the other hand, the *bphC* gene of another PCB-degrading strain, *Pseudomonas paucimobilis* Q1, lacked genetic as well as immunological homology with any of the other 15 biphenyl/PCB degraders tested. The existence of the nearly identical chromosomal genes among various strains may suggest that a segment containing the *bphABC* genes has a mechanism for transferring the gene from one strain to another.

Environmental contamination by polychlorinated biphenyls (PCB) was first reported in 1966. Since then, a number of PCB-degrading microorganisms have been isolated (1, 3, 4, 6, 9, 10, 13, 26). These PCB degraders are usually gram-negative soil bacteria which can utilize biphenyl as the sole source of carbon and energy. A few gram-positive strains have also been reported to degrade PCB (2, 21). These biphenyl-utilizing strains cometabolize PCB to chlorobenzoic acids by the major oxidative route as illustrated in Fig. 1 (6). Most of the biphenyl-utilizing strains cannot attack chlorobenzoic acids any further, so that the corresponding chlorobenzoates accumulate in the PCB catabolism. We cloned biphenyl-catabolic genes (*bph* genes) from one of the PCB degraders, *Pseudomonas pseudoalcaligenes* KF707 (11, 14). Three genes, *bphA* (encoding biphenyl dioxygenase), *bphB* (encoding dihydrodiol dehydrogenase), and *bphC* (encoding 2,3-dihydroxybiphenyl dioxygenase), were clustered (in that order) on the *Xho*I 7.2-kilobase (kb) DNA fragment (Fig. 1). We also cloned the chromosomal *bphC* gene from another PCB degrader, *Pseudomonas paucimobilis* Q1 (13). The 2,3-dihydroxybiphenyl dioxygenases (*bphC* gene products) from strains KF707 and Q1 are enzymatically very similar (7, 23): the molecular mass of native enzymes is 260 kilodaltons for both strains; the molecular mass of the subunit is 33 kilodaltons; the two enzymes contain ferrous ion as an essential cofactor; the structure of the holoenzymes is proposed to be $(\alpha FeII)_8$; and the enzymes are specific for 2,3-dihydroxybiphenyl but not for 3,4-dihydroxybiphenyl. The only difference in the enzymes is the specificity for catechol, which can be a substrate for the Q1 enzyme but not for the KF707 enzyme. Despite the enzymatic similarity, the *bphC* DNAs of KF707 and Q1 did not hybridize with each other, and the two enzymes showed no immunological cross-reactivity. Finally, we determined the nucleotide sequences of the two *bphC* genes (8, 23). The overall sequence homology of the 2,3-dihydroxybiphenyl dioxygenases at the amino acid level was as low as 38% between the two enzymes, but they possessed some homologous regions in which some amino acid sequences were significantly conserved (23). Recently, a *bph* gene cluster coding for the conversion of PCB to chlorobenzoic acid has been cloned from *Pseudomonas* sp. strain LB400 (20). The *bph* genes showed significant similarity with those of another PCB degrader, *Alcaligenes eutrophus* H850 (28).

We have isolated a number of biphenyl/PCB-degrading bacteria from various soils at different locations to investigate how the chromosomal *bph* genes are distributed, conserved, and regulated in various bacteria. Here we report on the molecular relationship of the *bph* genes and the immunological properties of 2,3-dihydroxybiphenyl dioxygenases among various biphenyl/PCB-degrading bacteria.

MATERIALS AND METHODS

Microorganisms and cultivation. Biphenyl-utilizing strains used in this study are listed in Table 1. *P. pseudoalcaligenes* KF707 was isolated from soil in Kitakyushu, Japan, as described previously (11). *P. paucimobilis* Q1 was isolated from soil in Chicago, Ill. (13). *Arthrobacter* sp. strain M5 was isolated in Chiba, Japan, and is the only gram-positive strain in our collection of biphenyl-utilizing strains (9). The other 12 strains were isolated from various locations in Japan, and they are all gram-negative soil bacteria. All 15 strains were grown with various carbon sources in a basal salts medium as described previously (9). For basal salts medium agar, biphenyl, 4-chlorobiphenyl, 4-methylbiphenyl, 2-bromobiphenyl, 2-nitrobiphenyl, 2-hydroxybiphenyl, or diphenylmethane was supplied as vapor on the lid of a

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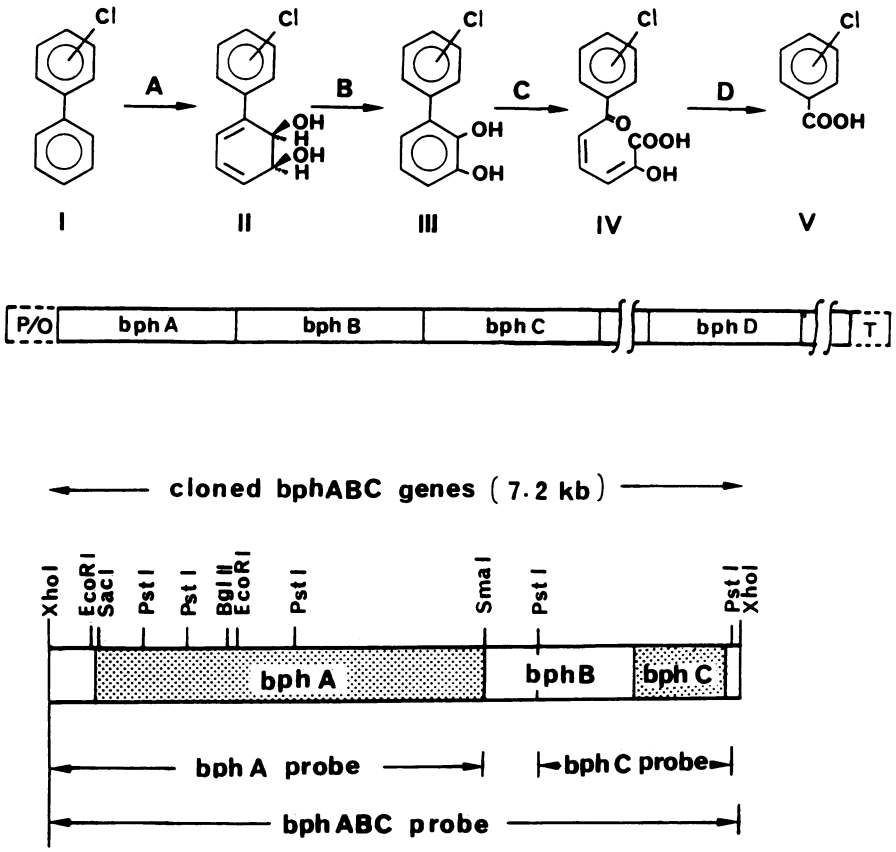

FIG. 1. (Top) Catabolic pathway for degradation of biphenyl and PCB by bacteria. The *bph* operon in *P. pseudoalcaligenes* KF707 consists of at least three genes (*bphA*, *bphB*, and *bphC*). Compounds: I, biphenyl; II, 2,3-dihydroxy-4-phenylhexa-2,4-diene (dihydrodiol compound); III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (meta-cleavage compound); V, benzoic acid. Enzyme activities: A, biphenyl dioxygenase; B, dihydrodiol dehydrogenase; C, 2,3-dihydroxybiphenyl dioxygenase; D, meta-cleavage compound hydrolase. Symbols: P/O, putative promoter-operator region; T, putative transcriptional terminator. (Bottom) DNA fragments used as probes in Southern blot hybridization.

petri dish. *Escherichia coli* JM109 (27), harboring a recombinant plasmid which contains the cloned *bphA*, *bphB*, and *bphC* genes from *P. pseudoalcaligenes* KF707, and *E. coli* C600 (18), harboring the cloned *bphC* gene of *P. paucimobilis* Q1, were grown in L broth (9).

Preparation of DNA, subcloning of *bph* genes, and Southern blot hybridization. Chromosomal DNAs from various biphenyl-utilizing strains were prepared essentially as described by Marmur (19). The plasmid carrying the 7.2-kb *Xho*I DNA fragment which contains the *bphA*, *bphB*, and *bphC* genes of *P. pseudoalcaligenes* KF707 was isolated as described previously (5). The 7.2-kb *Xho*I fragment was used as the *bphABC* probe (Fig. 1). The 4.5-kb *Xho*I-*Sma*I fragment which includes the entire *bphA* gene was used as the *bphA* probe. The 2.0-kb *Pst*I fragment which includes the *bphC* gene and part of the *bphB* gene was used as the *bphC* probe. The 2.6-kb *Xho*I DNA fragment carrying the *bphC* gene of *P. paucimobilis* Q1 was also used for hybridization (23). Hybridization experiments were performed by transferring DNA from agarose gels to Zeta-probe blotting membranes (Bio-Rad Laboratories, Richmond, Calif.). Hybridization with nick-translated [^{32}P]DNA was performed essentially as described by Southern (22).

Preparation of enzymes and immunological studies. 2,3-Dihydroxybiphenyl dioxygenases from various biphenyl-utilizing strains were prepared from the cells grown with biphenyl. The cells were disrupted by using a French pressure cell (Ohtake Co. Ltd., Tokyo, Japan) and centrifuged at 28,000 × *g* for 30 min. The supernatant fluids were used as a crude extract. The Ouchterlony immunological tests with rabbit antiserum raised against purified 2,3-dihydroxybiphenyl dioxygenase from either KF707 or Q1 were performed as described previously (23).

Western blots. The enzymes obtained from biphenyl-grown cells were treated with 25% sodium dodecyl sulfate, heated for 5 min at 95°C, and separated on 12% polyacrylamide stacking gels in a minigel apparatus (TEF Corp. Ltd., Tokyo, Japan). Western immunoblot experiments were carried out by using the Immun-Blot assay kits (Bio-Rad).

RESULTS

Isolation of biphenyl-utilizing strains. The 13 newly isolated strains were all gram negative, and they all utilized biphenyl as the sole source of carbon and energy. They converted 4-chlorobiphenyl to 4-chlorobenzoic acid and produced 2,3-dihydroxybiphenyl dioxygenase inducibly, so that their catabolism of biphenyl and PCB could be considered to proceed through the major oxidative route as shown in Fig. 1. The growth characteristics of 13 strains on various aromatic compounds are presented in Table 1, together with those of three other biphenyl-utilizing strains (*P. pseudoalcaligenes* KF707, *P. paucimobilis* Q1, and *Arthrobacter* sp. strain M5) that have been described previously (9, 11). Some

TABLE 1. Biphenyl-utilizing strains used and their growth characteristics on various biphenyl and benzoate derivatives

Strain	Growth[a] on following substrate:									
	BP	4ClBP	4MeBP	2BrBP	$2NO_2BP$	2OHBP	DM	BA	*m*-Tol	Sal
Achromobacter xylosoxidans KF701	+++	−	+	−	−	++	−	+++	−	++
Pseudomonas sp. strain KF702	+++	−	+	−	−	−	−	+++	−	−
P. fluorescens KF703	+++	−	−	−	−	−	+	+++	−	++
Moraxella sp. strain KF704	+++	−	−	−	−	−	+	+++	−	−
P. paucimobilis KF706	+++	−	+	+	+	−	++	+++	−	−
P. pseudoalcaligenes KF707	+++	−	+	−	−	−	−	+++	−	−
Alcaligenes sp. strain KF708	+++	+	++	++	++	−	++	+++	−	−
Unidentified strain KF709	+++	−	−	−	−	−	+	+++	−	++
Pseudomonas sp. strain KF710	+++	−	−	−	−	−	−	+++	−	−
Alcaligenes sp. strain KF711	+++	−	−	−	−	−	−	+++	++	−
Pseudomonas sp. strain KF712	+++	−	−	−	−	−	−	+++	−	−
P. stutzeri KF713	+++	−	−	−	−	−	−	+++	−	+
Pseudomonas sp. strain KF714	+++	+	+	−	−	−	+	+++	−	++
P. putida KF715	+++	+	+	−	+	++	+	+++	−	++
P. paucimobilis Q1	+++	+	+	−	−	−	+	+++	++	++
Arthrobacter sp. strain M5	+++	+	++	+	+	−	−	+++	+	−

[a] Growth was checked after 1 week of incubation at 30°C. Symbols: +++, good growth; ++, moderate growth; +, poor growth; −, no growth or very poor growth. Abbreviations: BP, biphenyl; 4ClBP, 4 chlorobiphenyl; 4MeBP, 4-methylbiphenyl; 2BrBP, 2-bromobiphenyl; $2NO_2BP$, 2-nitrobiphenyl; 2OHBP, 2-hydroxybiphenyl; DM, diphenylmethane; BA, benzoic acid; *m*-Tol, *m*-toluate; Sal, salicylate.

strains grew on various biphenyl derivatives. Strain KF708, for instance, grew on 4-chloro-, 4-methyl-, 2-bromo-, and 2-nitrobiphenyl, as well as on diphenylmethane.

DNA homology of *bph* gene cluster. Total cellular DNA isolated from each biphenyl-utilizing strain was digested with *Xho*I and then examined by Southern blot hybridization. When the *bphA* gene from strain KF707 was used as the probe (Fig. 1), homologous DNA segments were observed for 10 of 16 strains, including KF707 itself. Six strains (KF702, KF703, KF710, KF711, KF713, and KF714) had *bph* DNA on the 7.2-kb *Xho*I fragment and that was identical in size with KF707 *bph* DNA (data not shown). The other two strains, KF701 and KF715, carried homologous *bph* DNA on a 9.4-kb *Xho*I fragment. Two *Xho*I DNA fragments, of 6.0 and 2.5 kb, were hybridized in KF706. No significant DNA homology could be observed for KF704, KF708, JF709, KF712, *Arthrobacter* sp. strain M5, or *P. paucimobilis* Q1. Nearly identical results were obtained when the *bphC* DNA from KF707 was used as the probe (Fig. 1). In contrast to *bphABC* DNA from *P. pseudoalcaligenes* KF707, *bphC* DNA (*Xho*I 2.6-kb fragment) from *P. paucimobilis* Q1 did not hybridize with any DNAs from other biphenyl-utilizing strains, but only with Q1 genomic DNA (data not shown).

To examine the genetic organization of *bph* genes in more detail, the genomic DNAs from various biphenyl-utilizing strains were double digested with *Xho*I and *Pst*I. The KF707 *Xho*I 7.2-kb DNA fragment containing the *bphABC* genes was used as the probe. Since the KF707 *Xho*I 7.2-kb DNA fragment has five cutting sites for *Pst*I, six fragments (of 2.1, 1.9, 1.6, 0.9, 0.4, and 0.3 kb) hybridized with 7.2-kb *bphABC* (Fig. 2). A similar hybridization profile was observed for strains KF702, KF703, KF710, KF711, KF713, and KF714, except that 1.6 kb of DNA was missing in KF703. Strains KF701 and KF715 showed three bands (1.6, 0.9, and 0.4 kb) in common with KF707. A 6.5-kb DNA fragment was also hybridized in these two strains. KF706 showed four bands, but at different positions from the bands described above. These results indicated that the nucleotide sequences, or at least the restriction sites, within the *bph* genes of several biphenyl-utilizing strains were very similar, if not identical, to those of KF707. We then examined DNAs flanking the *bph* genes by digesting them with various endonucleases such as *Eco*RI, *Sal*I, and *Sma*I. Since the *Xho*I 7.2-kb DNA fragment from KF707 has two *Eco*RI cutting sites (Fig. 3), three DNA bands (6.5, 5.4, and 1.5 kb) hybridized with the *bphABC* probe. Two fragments (6.5 and 1.5 kb) were found in DNA from strains KF702, KF703, KF710, KF711, KF713, and KF714 when hybridized with the *bphABC* probe. The third DNA fragment, however, was hybridized in a different fashion for these six strains: 14 kb for KF702, 5.4 kb for KF703, 6.0 kb for KF710 and KF711, 7.5 kb for KF713, and 5.2 kb for KF714 (Fig. 3). These results imply that upstream outside DNA regions of the *bphA* gene seem to be occupied by unrelated DNA in these biphenyl-utilizing strains. There should be a common *Eco*RI site inside the *bph* operons of these seven strains in the downstream region of the *bph*C gene. Differences in the flanking regions of *bph* genes of KF701 and KF715 were also observed, since the 5.2-kb band observed in KF701 (Fig. 3, middle band) was

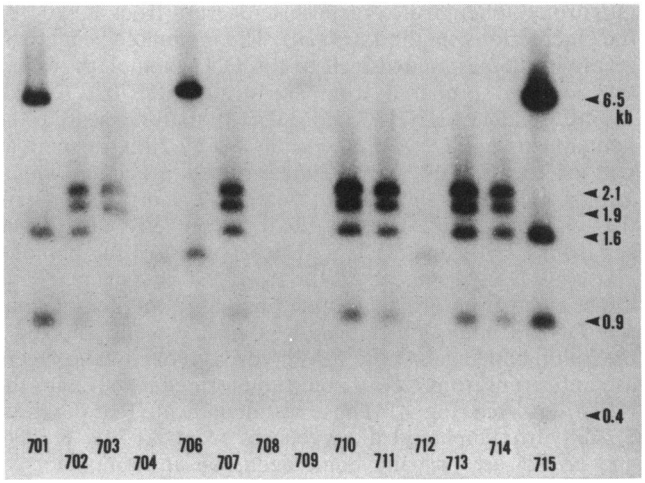

FIG. 2. Hybridization of KF707 *bphABC* genes with cellular DNAs of various biphenyl-utilizing strains. Total cellular DNAs were double digested with *Xho*I and *Pst*I, and the ^{32}P-labeled 7.2-kb *Xho*I fragment that includes the *bphABC* genes of KF707 (Fig. 1, bottom) was hybridized.

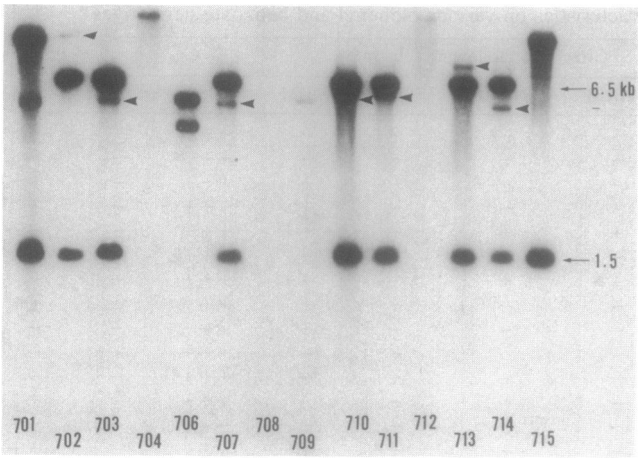

FIG. 3. Hybridization profiles of the various biphenyl-utilizing strain cellular DNAs which were digested with *Eco*RI. The probe was the same as in Fig. 2. Arrowheads (◀) indicate the *Eco*RI fragments of the upstream region of *bphA* in strains KF702, KF703, KF707, KF710, KF711, KF713, and KF714.

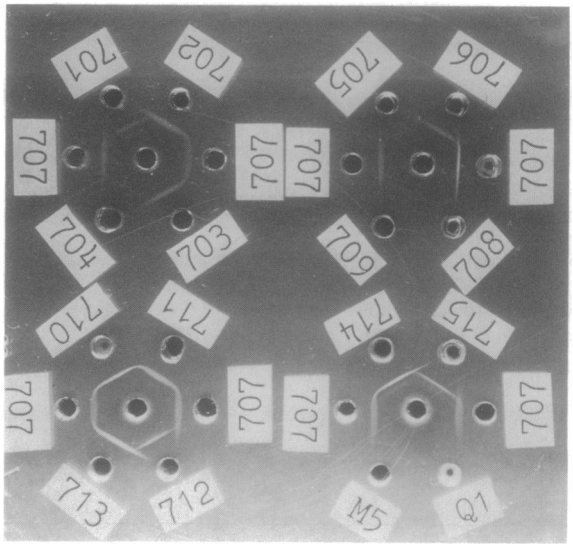

FIG. 4. Immunoprecipitin pattern of 2,3-dihydroxybiphenyl dioxygenases from various biphenyl-utilizing strains with antiserum prepared against the purified 2,3-dihydroxybiphenyl dioxygenase of strain KF707. The KF707 2,3-dihydroxybiphenyl dioxygenase antiserum was placed in the central wells. The crude cell extracts obtained from biphenyl-grown cells were placed in the wells surrounding the antiserum. In this figure, KF704 and KF705 are the same strain.

missing in KF715. Similar results, indicating a difference in the flanking regions of *bph* genes, were obtained when the genomic DNAs of the above eight strains were digested with *Sal*I or *Sma*I (data not shown). All 16 strains harbored one to three plasmids whose molecular sizes were up to ca. 150 kb, but the *bphABC* probe did not hybridize with any plasmid DNAs (data not shown).

Immunological analysis of 2,3-dihydroxybiphenyl dioxygenases. In a previous paper (23), we showed that despite the similarity of the 2,3-dihydroxybiphenyl dioxygenases in the two strains *P. pseudoalcaligenes* KF707 and *P. paucimobilis* Q1, these enzymes are immunologically distinct from each other. The same antisera raised against purified 2,3-dihydroxybiphenyl dioxygenases from strains KF707 and Q1 were used to examine the immunological cross-reactivity of 2,3-dihydroxybiphenyl dioxygenases from various biphenyl-utilizing strains. After growth with biphenyl to induce 2,3-dihydroxybiphenyl dioxygenase, the crude cell extracts were subjected to immunological analysis with the 2,3-dihydroxybiphenyl dioxygenase antiserum from KF707 by the Ouchterlony method. (Fig. 4). The immunological cross-reactivity corresponded well to the DNA homology. Thus, the enzymes prepared from six strains (KF702, KF703, KF710, KF711, KF713, and KF714) that possessed the homologous *bph* DNA on the same *Xho*I 7.2-kb fragment as that of the KF707 showed clear fused precipitin bands against the KF707 antiserum, without forming a spur with the KF707 enzyme. The 2,3-dihydroxybiphenyl dioxygenases from KF701 and KF715, which possessed the homologous *bph* DNA segment on the *Xho*I 9.4-kb fragment, and the enzyme from KF706, which possessed the homologous *bph* DNA segment on the *Xho*I 6.0-kb fragment, showed a precipitin band against the 2,3-dihydroxybiphenyl dioxygenase antiserum from KF707, but they formed a spur with the KF707 enzyme (Fig. 4). These results indicate that the three 2,3-dihydroxybiphenyl dioxygenases from KF701, KF706, and KF715 are partially homologous on an immunological basis with the KF707 enzyme. The 2,3-dihydroxybiphenyl dioxygenases from KF704 (KF705 is the identical strain to KF704 in Fig. 4) and KF712 also showed a weak precipitin band and formed a spur with the KF707 enzyme. The 2,3-dihydroxybiphenyl dioxygenases from KF708, KF709, M5, and Q1 showed no immunological cross-reactivity. On the other hand, the 2,3-dihydroxybiphenyl dioxygenase antiserum from Q1 did not cross-react with any enzymes from other biphenyl-utilizing strains except for the Q1 enzyme itself (data not shown).

Western blot analysis of 2,3-dihydroxybiphenyl dioxygenase. The denatured enzymes obtained from biphenyl-grown cells were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting analysis. The visualized enzyme subunits from strains KF701, KF702, KF703, KF710, KF711, KF713, KF714, and KF715 showed the same molecular mass (33 kilodaltons) as that of KF707 (Fig. 5).

DISCUSSION

Molecular relationships of some degradative plasmids have been extensively investigated. The catabolic genes of the meta pathway are highly conserved on the two toluene/xylene-catabolic plasmids, pWW0 and pWW53, although the basic replicon is very different (15). Molecular homology studies between *Pseudomonas* IncP9 degradative plasmids TOL, NAH, and SAL revealed that they have common DNA sequences (16). However, there are very few reports on the molecular homology of the chromosome-borne catabolic genes for xenobiotics. Therefore, in the present study, we collected and examined genes responsible for biphenyl/PCB degradation by various strains (a total of 16 strains) to find how chromosomal biphenyl-catabolic genes (the *bph* operon) are distributed and organized in various biphenyl-utilizing strains. To do this, we used *bph* genes of *P. pseudoalcaligenes* KF707 and *P. paucimobilis* Q1 as the probes. Our preliminary data for strain KF707 showed that the initial oxidation activity of biphenyl was lost if ca. 500 base pairs from the *Xho*I site in the direction of *bphC* gene was digested by exonuclease III (Fig. 1), implying that the KF707 *bphA* gene starts within 500 base pairs of the left

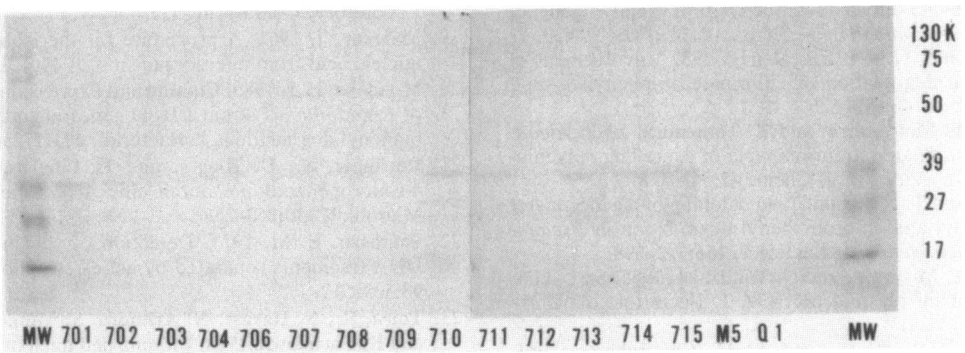

FIG. 5. Western blot of 2,3-dihydroxybiphenyl dioxygenases from various biphenyl-utilizing strains. Crude cell extracts of various biphenyl-utilizing strains grown with biphenyl were heated at 95°C for 5 min in the presence of 25% sodium dodecyl sulfate. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis the protein was electrophoretically transferred to the filter for the Western blot analysis. MW, Molecular weight standards (molecular weights are shown in thousands on the right-hand side).

*Xho*I site in Fig. 1. It was also shown that the *Xho*I-*Sma*I 4.5-kb DNA includes the entire *bphA* gene (14), so that the *bphA* cistrons may extend up to 4 kb. We used this *Xho*I-*Sma*I 4.5-kb DNA as the probe for the *bphA* gene. The *Pst*I 2.0-kb DNA, on the other hand, includes the entire *bphC* gene and part of the *bphB* gene, as evidenced from the previously sequenced fragment (8). The results show that biphenyl/PCB-degrading genes are chromosomally encoded in 15 strains and that some of them are nearly identical and some are very different. It should be noted that the KF707-type chromosomal *bph* genes are conserved widely in various biphenyl-utilizing strains. Although it is known that plasmid-encoded toluene/xylene-degrading genes on the TOL plasmid (pWW0) can be excised and transposed at several different positions (12, 17, 24, 25), to our knowledge this is the first report implying that chromosomal genes including the KF707-type *bph* operons might be mobilized to the other soil bacteria. We have started mixed-culture chemostat work to check whether the *bph* gene cluster of KF707 can be transferred to other soil pseudomonads.

Two strains, KF701 and KF715, possess homologous *bph* genes to those of KF707 on the 9.4-kb *Xho*I DNA fragment. We have recently cloned the 9.4-kb DNA from *P. putida* KF715, and the data indicate that the *bphD* gene (encoding hydrolase [Fig. 1]) is also located on the same 9.4-kb *Xho*I fragment in these two strains (N. Hayase, K. Taira, and K. Furukawa, submitted for publication).

In contrast to KF707 *bph* genes, the *bphC* gene of *P. paucimobilis* Q1 did not hybridize with any other biphenyl-utilizing strains tested, and no immunological cross-reactivity was observed for the Q1 2,3-dihydroxybiphenyl dioxygenase antiserum. It is noteworthy that *P. pseudoalcaligenes* KF707 was isolated in Japan, whereas *P. paucimobilis* Q1 was isolated in the United States. It is also true, though, that the enzymatic properties of 2,3-dihydroxybiphenyl dioxygenases of KF707 and Q1 are very similar in terms of their native as well as subunit molecular masses, cofactor, and enzyme activities. There are some highly conserved amino acid sequences that might function as binding domains for substrate, oxygen, and ferrous ion as the cofactor. Therefore, the dissimilarity of *bph* genes between strain Q1 and other biphenyl-utilizing strains may be seen only at the level of the nucleotide sequences (and hence the amino acid sequences); i.e., the tertiary structures of 2,3-dihydroxybiphenyl dioxygenases might be conserved among biphenyl-utilizing strains. In fact, the molecular mass of the subunit was the same (33 kilodaltons) from the Western blot analysis of strains KF707 and Q1 and other biphenyl-utilizing strains. This value is also the same for the only gram-positive strain, *Arthrobacter* sp. strain M5 (data not shown).

Thus, some biphenyl-catabolic genes are conserved, as in the chromosomal *bphABC* genes of *P. pseudoalcaligenes* KF707, among biphenyl-utilizing strains, but others are different. At present, our analysis can enable us only to surmise why these *bph* operons are distributed so widely and why some of them are highly conserved. Since there are biphenyl-related compounds in plant lignin, which is composed of many aromatic skeletons, such biphenyl/PCB-catabolic genes might originally have been involved in the degradation of certain components of decomposed lignin. If so, these genes could have evolved over a long period. The divergence of *bph* genes in biphenyl-utilizing strains might support this hypothesis.

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