J. Microbiol. Biotechnol. (2004), **14**(2), 302–311

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Versatile Catabolic Properties of Tn*4371*-encoded *bph* Pathway in *Comamonas testosteroni* (Formerly *Pseudomonas* sp.) NCIMB 10643

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Received: March 10, 2003
Accepted: June 16, 2003

Abstract *Comamonas testosteroni* (formerly *Pseudomonas* sp.) NCIMB 10643 can grow on biphenyl and alkylbenzenes (C_2-C_7) via 3-substituted catechols. Thus, to identify the genes encoding the degradation, transposon-mutagenesis was carried out using pAG408, a promoter-probe mini-transposon with a green fluorescent protein (GFP), as a reporter. A mutant, NT-1, which was unable to grow on alkylbenzenes and biphenyl, accumulated catechols and exhibited an enhanced expression of GFP upon exposure to these substrates, indicating that the *gfp* had been inserted in a gene encoding a broad substrate range catechol 2,3-dioxygenase. The genes (2,826 bp) flanking the *gfp* cloned from an *Sph*I-digested fragment contained three complete open reading frames that were designated *bphCDorf1*. The deduced amino acid sequences of *bphCDorf1* were identical to 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD), and Orf1, respectively, that are all involved in the degradation of biphenyl/4-chlorobiphenyl (bph) by *Ralstonia oxalatica* A5. The deduced amino acid sequence of the *orf1* revealed a similarity to those of outer membrane proteins belonging to the OmpW family. The introduction of the *bphCDorf1* genes enabled the NT-1 mutant to grow on aromatic hydrocarbons. In addition, PCR analysis indicated that the DNA sequence and gene organization of the *bph* operon were closely related to those in the *bph* operon from Tn*4371* identified in strain A5. Furthermore, strain A5 was also able to grow on a similar set of alkylbenzenes as strain NCIMB 10643, demonstrating that, among the identified aromatic hydrocarbon degradation pathways, the *bph* degradation pathway related to Tn*4371* was the most versatile in catabolizing a variety of aromatic hydrocarbons of mono- and bicyclic benzenes.

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Key words: Tn*4371*, *bph* operon, *Comamonas testosteroni* NCIMB 10643, GFP tagging, alkylbenzenes, OmpW

Many studies have already been carried out on the bacterial aerobic degradation of monoalkylbenzenes. In most cases, these aromatic hydrocarbons are degraded through the formation of the first intermediate of *cis*-dihydrodiol by the action of Rieske non-heme iron oxygenases [17]. The metabolite is then converted to a catechol for the subsequent *meta*-cleavage reaction [18, 47]. The toluene degradation (*tod*) pathway in *Pseudomonas putida* F1 [16] is one of the well-characterized pathways for the aerobic bacterial catabolism of 1-substituted benzenes and consists of seven enzymatic reactions for the conversion of benzene, toluene, and ethylbenzene into pyruvate and acetyl-CoA [5, 29, 30, 56].

Some microorganisms use biphenyl and/or 1-alkykylbenzenes as growth substrates, using enzyme systems similar to those in the *tod* pathway. Despite having similar chemical structures, in many cases, 1-alkylbenzenes and biphenyl are not used as growth substrates by a single degradation pathway. For instance, a previous experiment conducted by the current authors showed that the biphenyl-degrader *Burkholderia* sp. LB400 [34] is incapable of growing on benzene, *n*-alkylbenzenes (C_1-C_{12}), or isopropylbenzene. In addition, the biphenyl-degrader *Pseudomonas pseudoalcaligenes* KF707 is known for its inability to grow on benzene, toluene, and ethylbenzene [14]. In contrast, it has been reported that the alkylbenzene-degraders *P. putida* F1 [16], *P. putida* RE204 [10], *Rhodococcus* sp. DK17 [26], and *Pseudomonas fluorescens* IP01 [19] are unable to grow on biphenyl, indicating that the induction and/or activities of the pathway

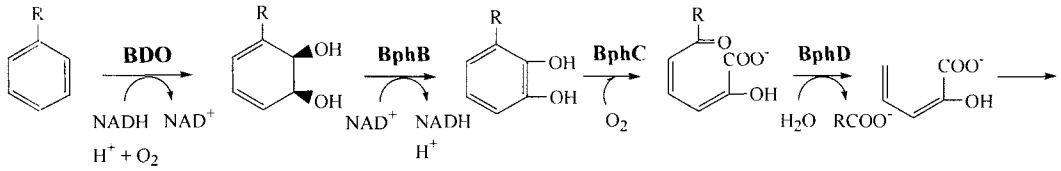

Fig. 1. Early steps of degradation pathway for 1-alkylbenzenes and biphenyl in strain NCIMB 10643.
Enzymes: BDO, biphenyl 2,3-dioxygenase; BphB, biphenyl *cis*-2,3-dihydrodiol dehydrogenase; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase.

enzymes in these microorganisms could have discrete metabolic specificities for mono- and bicyclic benzenes.

Pseudomonas sp. NCIMB 10643 was originally isolated in England by Evans' group due to its ability to grow on biphenyl [31], plus it was proven to degrade biphenyl into benzoate by a pathway similar to the *tod* pathway [49]. The strain was also demonstrated to grow on a range of 1-alkylbenzenes (linear with C_2-C_7 and isopropylbenzene, isobutylbenzene, *sec*-butylbenzene, *tert*-butylbenzene, and *tert*-pentylbenzene) with a *meta*-aromatic compound degradation pathway [48] (Fig. 1). Therefore, NCIMB 10643 is one of the most versatile 1-substituted benzene-degrading bacterial strains reported thus far. Although the biochemical studies on the biodegradation pathway were carried out in detail thirteen years ago by Smith and Ratledge, genetic information on the pathway is still unavailable. Accordingly, the current study used a mini-Tn*5* transposon with a green fluorescent protein (GFP)-based reporter system, pAG408 [52] to identify the genes responsible for the degradation of alkylbenzenes and biphenyl by strain NCIMB 10643.

MATERIALS AND METHODS

Materials

Most of the chemicals used in the current study were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), with the exception of isopropyl-β-D-thiogalactopyranoside (IPTG; Duchefa, Haarlem, The Netherlands) and 2,3-dihydroxybiphenyl (Wako Pure Chemicals, Japan). All chemicals were of analytical grade. Strain NCIMB 10643 was obtained from NCIMB Ltd. (A Berdeen, U.K.). The enzymes used for the nucleic acid manipulation were purchased from KOSCO (Kyungki, Korea), Promega (Madison, WI, U.S.A.), and Gibco BRL (Gaithersburg, MD, U.S.A.). Strain F1 was provided by Dr. David T. Gibson (University of Iowa). The *Ralstonia oxalatica* A5 [46] was provided by Dr. Sung-Cheol Koh (Korea Maritime University, Pusan, Korea) and pPROBE-GT [33] provided by Dr. Steven E. Lindow (University of California, Berkeley).

Bacterial Strains and Culture Conditions

Strain NCIMB 10643 was grown in a Luria-Bertani (LB) or minimal salts medium (MSB) [51] with an appropriate carbon and energy source. Alkylbenzenes were supplied in the vapor phase to support the growth of strain NCIMB 10643. *E. coli* DH5α was used as the host organism for plasmid retention, and was grown in LB. Ampicillin (50 μg/ml) or kanamycin (25 μg/ml) was used for growth of the transformed DH5α cells. Succinate (10 mM), and kanamycin (0.2 mg/ml), or gentamycin (0.5 mg/ml) were then used to grow the transformed NCIMB 10643 cells.

Isolation of Growth-Defective Mutants on Aromatic Hydrocarbons

The introduction and subsequent transposition of the mini Tn*5* transposon into the genome of strain NCIMB 10643 were carried out by mating strain NCIMB 10643 with *E. coli* S17-1(pAG408) [52] as previously described [5]. Transconjugants impaired in catechol 2,3-dioxygenase were selected on MSB agar containing succinate, kanamycin, and propylbenzene supplied in the vapor phase. After 5 days of incubation at 30°C, colonies producing a brown pigment were selected. One of the mutants, designated NT-1, produced large amounts of catechols when provided with various aromatic hydrocarbons that support the growth of the wild-type strain, and also only expressed GFP upon exposure to these chemicals. As such, this NT-1 mutant was selected for further study.

Determination of Metabolites Accumulated by NT-1

Mutant NT-1 was grown for 24 h at 28°C with shaking at 180 rpm in MSB liquid with 10 mM succinate and propylbenzene supplied as a vapor. The cells were harvested aseptically by centrifugation and the cell pellets were stored at -72°C until used. The frozen cells were suspended to an OD_{600} of 1.0 in 100 ml of MSB medium in 250-ml Erlenmeyer flasks that contained 10 mM succinate. Aromatic hydrocarbons dissolved in methanol were directly added to the flasks to a final concentration of 1 mM. The biotransformations were carried out at 28°C with shaking at 180 rpm for 2 days. The pH of the culture supernatants was adjusted to 2-3 with HCl, then the supernatants were extracted with ethyl acetate and concentrated as described previously [4]. A GC/MS analysis was carried out under the same conditions as described previously [4].

Cloning and Identification of *gfp*-containing DNA Fragment from Mutant NT-1

The chromosomal DNA from strain NT-1 was prepared as described previously [44]. The plasmids were isolated using a Bioneer miniprep kit (Taejeon, Korea). For shotgun cloning, the genomic DNA was digested using *Sph*I and ligated into pUC19 [55] that had been treated with *Sph*I and alkaline phosphatase, then the resulting plasmids were introduced into competent *E. coli* DH5α cells by transformation [44]. The transformants were selected on LB plates containing kanamycin. In addition, the transformants carrying the *gfp* gene were identified under a UV lamp at 362 nm. The plasmid DNA from one of the DH5α clones with the brightest GFP intensity was identified and named pJS02.

DNA Sequence Analysis

Plasmid pJS02 was used as the template for the DNA sequencing. The nucleotide sequences were determined by Genotech Co. (Daejeon, Korea) using an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.) with M13 and sequence-based primers. The searches for specific nucleotide or amino acid sequences were carried out using the BLAST program [1] provided by the National Center for Biotechnology Information (NCBI) and ExPASy Interface to EMBnet-CH/SIB/CSCS provided by the Swiss Institute of Bioinformatics (SIB) on the web pages http://www.ncbi.nlm.nih.gov/BLAST and http://www.expasy.org/cgi-bin/BLASTEMBnet-CH.pl, respectively. The nucleotide sequence of the partial 16S rDNA gene of strain NCIMB 10643 was determined by direct sequencing of the PCR product amplified using the 27F and 1522R primers [23] with Ex-Taq DNA polymerase (TaKaRa, Japan).

PCR Amplification

The reaction mixtures (50 μl) contained chromosomal DNA (20 ng), ExTaq DNA polymerase (1 U), dNTP (0.2 mM each), and the primer set (0.5 μM each) in the buffer supplied by the manufacturer. The PCR was carried out using a Bioneer thermal cycler (Taejeon, Korea) under the following conditions: 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 3 min at 72°C, and 5 min at 72°C. The primers as shown in Fig. 4. were designed based on known sequences for the *bph* regions in Tn*4371* from strain A5 [32, 36]. The primers (43-CF and 43-QR) were synthesized by the Bioneer Co and used to amplify the *bphCDorf1* genes. The electrophoresis was carried out using a 20 μl reaction volume on a 1.2% agarose gel with TAE buffer [44]. The nucleotide sequences (5'->3') of the primers were as follows: II-f, CAT ACG GGC AGC GTG TGA TC; II-r, CAA GGA GGT CAG TCC GAT CTT G; III-f, CAA GAT CGG ACT GAC CTC CTT G; III-r, CAG CCA GGA GCG TGC AAA GAC; IV-f, TCT TTG CAC GCT CCT GGC TG; IV-r, AGG TAA CCC AAA CGT TCG ATG; V-f, CAT CGA ACG TTT GGG TTA CCT C; V-r, CCC ATG AAG TCT TTA GAC AAG; VI-f, TTG TCT AAA GAC TTC ATG GGC; VI-r, CGG TAT GGT GTA TTG CGT TTG; 43-CF, GCC TAT GTG TTC TTT GCC ACG CGC; 43-QR, CGA TTA CCG TAC GTT ATC GAG CCG.

Construction of Plasmids and Recombinant Strains

Figure 2 shows the restriction enzyme sites used for the plasmid construction. The pJS02 plasmid was digested with *Sac*II and self ligated with T4 DNA ligase. The resulting plasmid (pJS022) was digested using *Sma*I and self-ligated to yield a *bphD* expression plasmid, pJS023. In order to test the promoter activity in the intergenic sequence between *bphD* and *orf1*, the *Nru*I-*Eco*RI fragment (1 kb) was ligated to a broad-host-range promoter-probe vector using a GFP reporter, pPROBE-GT, digested with *Sma*I and *Eco*RI. The resulting plasmid (pJS024) was used to transform competent *E. coli* S17-1 cells and then introduced into strain NCIMB 10643 by conjugation. The transconjugants were selected on MSB agar containing succinate, ampicillin, and gentamycin.

The PCR fragment (2.8 kb) obtained from amplifying the chromosomal DNA of NCIMB 10643 with primers 43-CF and 43-QR was ligated to a PCR product cloning T-vector, pEZ-T (RNA Co., Korea). This resulting vector (pJS041) was then amplified in *E. coli* DH5α and digested using *Sac*I and *Kpn*I. Next, the PCR-amplified insert was ligated to a broad-host-range vector pBBR1MCS-2 [28] and the resulting plasmid (pJS042) introduced into strain NT-1 by conjugation. The transconjugants were selected on MSB agar containing succinate, ampicillin, and kanamycin, or MSB agar containing kanamycin with a vapor supply of *n*-propylbenzene.

Preparation of Cell Extracts and Enzyme Activity Assays

The methods used for the IPTG-induction of the cloned gene from the recombinant *E. coli* cells and preparation of cell extracts from the bacterial cells were described in a previous report [4]. The *meta*-cleavage products were made as previously described [4] in the presence of 0.1 M potassium phosphate (pH 7.5). The activities of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (6-phenyl-HOHD) hydrolase (BphD) were determined at 25°C by measuring the absorbance decrease of each *meta*-cleavage product as previously described [5]. The enzyme-specific activities are reported as the micromoles of substrate utilized per minute per milligram of protein. The 2,3-Dihydroxybiphenyl 1,2-dioxygenase (BphC) activity of NT-1 was measured using cell extracts with 2,3-dihydroxybiphenyl as the substrate. The formation of ring fission product was monitored at a wavelength of 434 nm.

Fluorescence Measurements

The mutant NT-1 was grown with shaking, as described above, in MSB medium containing 10 mM succinate for 1

day. Inoculation (2 ml, final OD_{600} of 0.05) was made in fresh MSB medium containing 10 mM succinate with volatile compounds supplied as vapor (50 µl) or with the direct addition of solid chemicals to a final concentration of 1 mM. The medium volume was 50 ml in 250-ml Erlenmeyer flasks. The cells were harvested after 36 h of inoculation by centrifugation, washed twice with saline, and resuspended in saline at an OD_{600} of around 0.2. The intensity of the fluorescence was measured using a spectrofluorophotometer (model RF-5391PC, Shimadza Co.) as previously described [5].

Nucleotide Sequence Accession Numbers

The DNA sequences obtained in the current study are available from GenBank under accession numbers AY247415 and AF468021.

RESULTS

Reclassification of Strain NCIMB 10643

The almost complete 16S rDNA sequence (1,452 bp, GenBank accession number AY247415) of strain NCIMB 10643 exhibited the highest sequence identity (99%) to the equivalent genes of *Comamonas testosteroni* MBIC3841 (AB007997) and MBIC3840 (AB007996). The biochemical and physiological properties obtained using an API20NE kit revealed that the test strain had a 99.5% homology with *C. testosteroni*, indicating that the NCIMB 10643 strain should be reclassified as *C. testosteroni*.

Characterization of Aromatic Hydrocarbon-Negative Mutant, NT-1

The isolated mutant, NT-1, was unable to grow on selected aromatic hydrocarbons, including linear alkylbenzenes (C_2-C_7), isopropylbenzene, isobutylbenzene, *sec*-butylbenzene, *tert*-butylbenzene, and biphenyl, all of which are growth-supporting carbon sources for strain NCIMB 10643 [49]. The strain also accumulated brown to reddish-brown pigments when aromatic hydrocarbons were supplied in the vapor phase after growth on LB or MSB-succinate agar or in liquid cultures. In addition, other aromatic hydrocarbons, such as benzene, toluene, *n*-alkylbenzenes (C_8, C_9, C_{10}), and 4-chlorobiphenyl were transformed into compounds exhibiting a light brown pigment (Table 1). When the liquid culture was centrifuged, most of the pigments, predicted to be polymerized products of catechols, were precipitated with the cells.

In addition, the mutant also expressed GFP in MSB-succinate medium in the presence of various aromatic compounds (Table 1). The expression was 10- to 40-fold higher in the presence of the growth-supporting aromatic hydrocarbons for NCIMB 10643. Increased induction levels (approximately 35-fold) were also recorded in the presence 2,3-dihydroxybiphenyl. These results indicate that the *gfp*-based promoter-probe mini-transposon from pAG408 was inserted within an inducible gene involved in aromatic hydrocarbon degradation in strain NCIMB 10643.

To analyze the metabolite(s) accumulated during the exposure to aromatic hydrocarbons, biotransformations of the aromatic hydrocarbons were carried out using NT-1

Table 1. Induction of catechol formation and expression of fluorescence by various chemicals from strain NT-1.

Chemical	Growth of NCIMB 10643[a]	Catechol formation by NT-1[b]	Specific fluorescence expression by NT-1
No chemical	0	-	5.2±0.0
Benzene	0	+	20.2±0.2
Toluene	0	+	21.2±0.5
Ethylbenzene	1	+++	214.3±9.1
Propylbenzene	1	+++	224.4±6.6
Isopropylbenzene	1	+++	188.3±6.4
n-Butylbenzene	1	+++	177.9±12.4
n-Pentylbenzene	1	+++	175.0±14.0
n-Hexylbenzene	1	++	50.5±0.8
n-Heptylbenzene	1	++	47.3±0.7
n-Octylbenzene	0	++	36.7±0.8
n-Nonylbenzene	0	++	29.6±0.6
n-Decylbenzene	0	+	26.6±0.9
Chlorobenzene	0	+	15.4±0.1
Biphenyl	1	+++	215.3±5.7
2,3-Dihydroxybiphenyl	1	NA[c]	181.3±6.6
Indole	0	-	35.8±1.1
Naphthalene	0	-	7.5±0.3
4-Chlorobiphenyl	0	+	45.6±0.4

[a] 1, growth; 0, no growth.
[b] Color formation on MSB-succinate in presence of test chemical. +++, deep brown; ++, brown; +, lighter brown; -, no color.
[c] Not applicable.

resting cell systems as described in Materials and Methods. When the ethyl acetate extracts of the biphenyl transformations were analyzed by GC-MS, only one product peak was obtained. The compound exhibited a retention time of 21.07 min and its mass fragmentation pattern was identical to that generated by authentic 2,3-dihydroxybiphenyl. Both benzene and *n*-alkylbenzenes (C_1-C_{10}) yielded a major product with a molecular ion expected to be a catechol [$(M_{hydrocarbon}+32)^+$]. In addition, minor amounts (less than 5% of the total products) of benzylic monooxygenation products [4] were produced from *n*-alkylbenzenes with a side chain length longer than C_4. This indicates that NT-1 was possibly mutated in a gene encoding a catechol 2,3-dioxygenase, and the aromatic hydrocarbons were degraded to catechols. In addition, the cell extracts of propylbenzene-induced NT-1 exhibited no detectable dioxygenase activity toward 2,3-dihydroxybiphenyl.

Cloning of *gfp*-inserted Catechol Dioxygenase and Its Flanking Genes

The shotgun cloning of the *Sph*I-digested DNA fragment containing the *gfp* gene from NT-1 into pUC19 yielded two types of clones with either a very faint or strong GFP expression in *E. coli*. Nine transformants were selected for plasmid purification and a restriction analysis. All selected transformants contained the same 5.3 kb insert in pUC19, indicating that the mini-transposon was inserted at one specific site in the chromosome of strain NCIMB 10643. The different level of GFP expression in the recombinants was found to be due to the orientation of the insert with respect to the pUC19-derived *lac* promoter. Two different expression clones, DH5α (pJS01) and DH5α(pJS02), were selected and the level of GFP expression measured after 24 h of culturing in LB. The specific GFP expression level in DH5α (pJS02) was about 9-fold higher than that in DH5α (pJS01).

Nucleotide Sequence Analysis and Its Deduced Proteins

The nucleotide sequence of the *Sph*I insert (2,826 bp, GenBank accession number AF468021) in pUC19 flanking the mini Tn5 transposon was almost identical to the *bph* genes in Tn*4371* from strain A5 (AJ536756), which encode genes for the degradation of (4-chloro)biphenyl; a noncoding region included one base deletion and one base addition. This comparison and database search allowed the identification of four open reading frames, a partial *bph*B, and complete *bph*C, *bph*D, and *orf1* from the cloned DNA sequence (Fig. 2). The *bphB*, *bphC*, and *bphD* genes encode biphenyl *cis*-2,3-dihydrodiol dehydrogenase (BphB), BphC, and BphD, respectively. The *orf1* gene is not known to have any definite function in Tn*4371* and the result of further sequence comparisons is included in the following section. A sequence analysis revealed that the transposon was

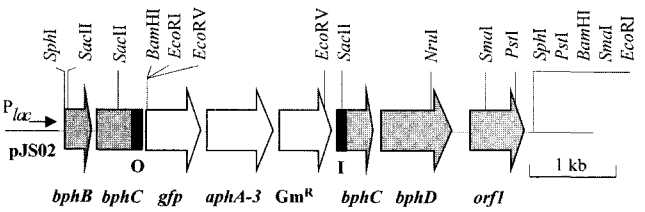

Fig. 2. Genetic organization and restriction endonuclease map of insert in pJS02.
The names of the *bph* genes with filled arrows are represented in Fig. 1. The open arrows originate from pAG408: *gfp*, *aphA-3* and Gm^R genes encoding GFP, 3-aminoglycoside phosphotransferase and gentamycin resistance, respectively. I and O represent the inner and outer ends of IS*50*, respectively. The points of the arrows indicate the direction of transcription.

inserted in the middle of *bph*C (at a position corresponding to Arg193 in the protein) with a repeat of 9-bp (5'-GCAATGGGC-3') directly flanking the borders of Tn*5*. The nucleotide sequence of strain NCIMB 10643 also showed a high degree of identity to those of the PCB-degraders *C. testosteroni* TK102 (AB086835, 94% in 2,063-bp) [20], *Achromobacter georgiopolitanum* KKS102 (M26433, 91.8% in 1,965-bp) [13, 25], and *Pseudomonas* sp. SY5 (AF190706, 96.2% in 882-bp; AF190707, 92.3% in 861-bp) [37]. Thus, it was concluded that the *bphBCDorf1* genes identified from NCIMB 10643 belong to the Tn*4371*-related biphenyl-degrading gene family. This result also indicates that the genes were less closely related to genes identified from alkylbenzene-degrading bacteria such as strains RE204 [10], IP01 [19], JR1 [42], and F1 [56].

Orf1

The deduced amino acid sequence of the *orf1* (218 residues, 22.7 kDa) was most closely related to DoxH/PahQ and NahQ (around 30% identity and 50% similarity, respectively) that are found in the polycyclic aromatic hydrocarbon and dibenzothiophene degradation operons [8, 9, 12, 45]. A 21-23% amino acid sequence homology was also found with a group of OmpW outer membrane proteins including the AlkL from the OCT-plasmid-encoded alkane degradation operon [54], Omp21 from *Comamonas acidovorans* [3], and OmpWs from *E. coli* [43], *Vibrio cholerae* [22], and *P. aeruginosa* PAO1 [15]. The DAS program (http://www.sbc.su.se/~miklos/DAS) [6] indicated that the *N*-terminus of Orf1 (first 22 residues) contained a transmembrane segment (Fig. 3), while the SignalP program (http://www.cbs.dtu.dk) [38] predicted that the cleavage site would be at position 22. As such, these results indicate that the Orf1 was an outer membrane protein in which the first 22 residues likely function as a signal sequence. In addition, the first 127 amino acids of the Orf1 from NCIMB 10643 were 79% identical to those of the Orf1 from strain KKS102 (total 251 residues) [24], yet the remainder of the sequence differed significantly,

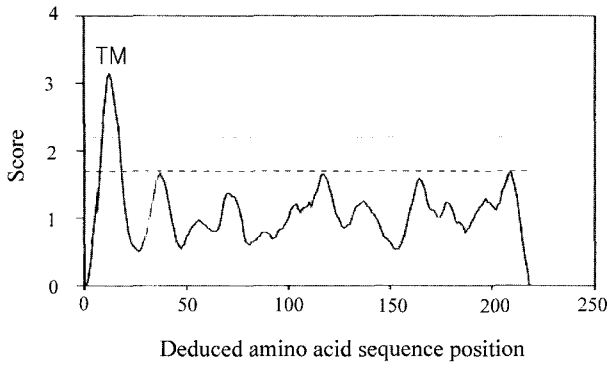

Fig. 3. Predicted transmembrane domain of Orf1 using DAS program.
The lower broken and upper straight lines are the loose and strict cutoffs, respectively.

implying that the *orf1*s with an unassigned function found in the *bph* gene clusters from strains KKS102 and A4 could encode an outer membrane protein belonging to the OmpW family.

Gene Expression Analysis and Complementation of NT-1 Mutant

No BphD activity (see below) was detected in the cell extracts prepared from strains DH5α (pJS02) and NT-1, indicating that the polar effect caused by the insertion of the transcriptional terminator in the mini-transposon affected the expression of *bphD*. When the vector pJS024, which contains a transcriptional fusion between a GFP reporter gene and the 240 bp DNA fragment located upstream of the *orf1* (Fig. 2), was introduced into strain NCIMB 10643, the resulting recombinant strain was unable to express GFP upon exposure to propylbenzene. Therefore, these two results indicate that *bphCDorf1* may have been co-transcribed.

Furthermore, when the genes encoding BphCDOrf1 were introduced into mutant NT-1, the resulting strain NT-1 (pJS042) was able to grow as fast as strain NCIMB 10643 on the aromatic hydrocarbon growth substrates of the wild-type strain, demonstrating that the *bph* operon was responsible for the degradation of monoalkylbenzenes and biphenyl in strain NCIMB 10643.

Substrate Preference of $BphD_{NCIMB\ 10643}$

The degradation of aromatic hydrocarbons is sometimes limited by the hydrolysis of the derivatives of HOHD [5, 14]. Thus, to determine the substrate preference of the hydrolase, a *bphD* expression vector was constructed and expressed in *E. coli* as described in Materials and Methods. The cell extract prepared from strain DH5α (pJS023) was incubated with the *meta*-cleavage products of catechol and substituted catechols. The preferred order of specificity was the *meta*-cleavage products of 3-phenylcatechol, 3-isopropylcatechol, 3-propylcatechol, 3-methylcatechol, and catechol with a specific activity of 825±122, 626±8, 386±11, 80±6, and 9.4±0.2 mU, respectively. BphD showed no measurable activity toward the *meta*-cleavage products of 4-methylcatechol. Although the specific activities were different, this result agreed with previously determined

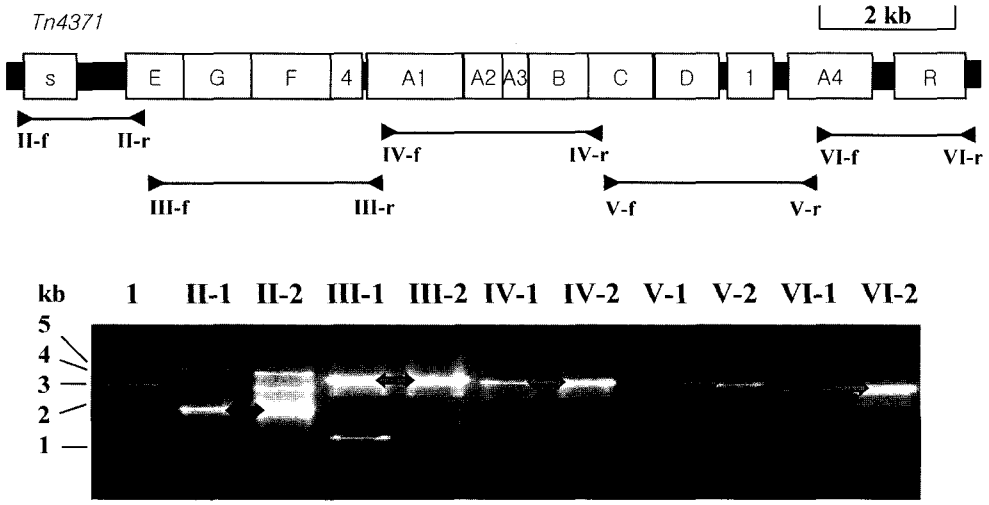

Fig. 4. PCR analysis showing high sequence relatedness between *bph* operons from strains A5 and NCIMB 10643.
(Top panel), genetic organization of *bph* regions of Tn*4371* from reference [50]; (Middle panel), location of PCR primers in Tn*4371* with indicated amplification lengths; (Bottom panel), comparison of PCR products formed from chromosomes of strains A5 and NCIMB 10643: lane 1, 1 kb ladder (ATgene, Korea); lane II-1, A5 amplified with II-f and II-r; lane II-2, NCIMB 10643 amplified with II-f and II-r; lane III-1, A5 amplified with III-f and III-r; lane III-2, NCIMB 10643 amplified with III-f and III-r; lane IV-1, A5 amplified with IV-f and IV-r; lane IV-2, NCIMB 10643 amplified with IV-f and IV-r; lane V-1, A5 amplified with V-f and V-r; lane V-2, NCIMB 10643 amplified with V-f and V-r; lane VI-1, A5 amplified with VI-f and VI-r; lane VI-2, NCIMB 10643 amplified with VI-f and VI-r. The expected sizes of the PCR products are shown by arrows.

from the cell extract of strain NCIMB 10643 [48] and further indicates that the cloned *bphD* gene was responsible for the degradation of both alkylbenzenes and biphenyl in strain NCIMB 10643. The expression of *bphD* was also analyzed by SDS-polyacrylamide gel electrophoresis, and BphD with a molecular mass of 32 kDa was identified from a standard curve of size markers.

Relatedness of *bph* Degradation Pathways in Strains NCIMB 10643 and A5

As mentioned above, the DNA sequence from strain NCIMB 10643 cloned in pJS02 exhibited almost the same identity to that in Tn*4371* from strain A5. In order to further characterize the DNA sequence relatedness of the genes encoding the degradation pathway enzymes in two strains, PCR analysis was carried out using primers in which the oligonucleotide sequences were designed to cover the whole *bph* region in Tn*4371*. PCR products with expected sizes were obtained from both strains, A5 and NCIMB 10643 (Fig. 4), indicating that the DNA sequence and genetic organization of the *bph* operon in strain NCIMB 10643 were both highly similar to the *bph* region of Tn*4371*.

This result also suggests that strain A5 could accommodate alkylbenzenes as pathway substrates using the *bph* gene products encoded in Tn*4371*. When the growth of strain A5 was tested in MSB agar, it seemed to grow on selected aromatic hydrocarbons, such as *n*-alkylbenzenes with a side chain length of C_3-C_8, but it was unable to grow on benzene, toluene, ethylbenzene, and other *n*-alkylbenzenes with a side chain length greater than C_8. Although the possibility of the presence of another degradation pathway for alkylbenzenes in strain A5 cannot be ruled out at present, the results obtained from the DNA sequence comparison and PCR analysis suggest that the *bph* pathway enzymes from Tn*4371* were also involved in the degradation of the alkylbenzenes. In contrast, 4-chlorobiphenyl, which is a growth substrate for A5, was unable to support the growth of strain NCIMB 10643.

Discussion

The *cis*-dihydrodiol degradative pathways for monoalkylbenzenes can be characterized based on their group of growth substrates and genetic sequence homology among Gram-negative bacteria. The first pathway is the benzene-toluene-ethylbenzene degradation pathway found in *P. putida* strains F1 [16], DOT-T1 [35], and CE2010 [39]. The second pathway uses toluene, ethylbenzene, isopropylbenzene, and *n*-butylbenzene, but not benzene or *n*-hexylbenzene, as shown by strain RE204 [11]. This pathway was originally identified from bacterial isopropylbenzene degradation. Similar strains include strains JR1 [42] and IP01 [19]. The third pathway found in strain NCIMB 10643 uses a broader range of 1-alkylbenzenes than the first two pathways mentioned, plus it is the only pathway that can utilize biphenyl, giving it the most relaxed range of catabolizable substrates. Unlike the first two pathways, genetic information on the catabolism of strain NCIMB 10643 has been unavailable until now.

Accordingly, the current study used a promoter-probe mini-transposon with GFP as a reporter to identify the (*bph*) genes encoding enzymes for alkylbenzene and biphenyl degradation in strain NCIMB 10643. This reporter system was then used to determine the transcriptional activity of the catabolic operon in response to various chemicals. A selected mutant, NT-1, was shown to have the GFP reporter gene inserted in the appropriate orientation in a gene encoding catechol dioxygenase. When the growth substrates of the wild-type strain were converted into 3-substituted catechols, they increased the induction of GFP in strain NT-1 (Table 1). To a lesser extent, benzene, toluene, *n*-alkylbenzenes (C_8, C_9, C_{10}), and 4-chlorobiphenyl also induced catechol formation and GFP expression, indicating that the regulation of the operon was not tightly controlled. This leaky control mechanism occurs in several degradation pathways and could be an advantage for the adaptation of the strain to new environments including xenobiotic aromatic compound(s) with similar structures [7, 41]. The induction of GFP in the NT-1 mutant by 2,3-dihydroxybiphenyl was the same level as that induced by biphenyl, a growth substrate of strain NCIMB 10643. In addition, NT-1 had a negligible *meta*-cleavage activity. Therefore, these results may imply that 3-substituted catechols could be inducers of *bph* operons. A previous study using strain A5 excluded the possibility that biphenyl or 4-chlorobiphenyl by itself could induce a *bph* operon [36]. Thus, the possible involvement of catechols as the inducers of a *bph* operon is intriguing, as the biphenyl degradation pathway in strain KKS102, the genes of which have a high sequence homology to the *bph* genes, is induced by 6-phenyl-HOHD [40].

The genes flanking the transposon insertion in the mutant NT-1 were cloned from *Sph*I-digested chromosomal DNA fragments. Complete genes encoding a 3-substituted catechol dioxygenase (BphC), 6-substituted HOHD hydrolase (BphD), and outer membrane protein (Orf1) were identified. The genes were most closely related to those involved in the degradation of biphenyl and 4-chlorobiphenyl from strains A5, KKS102, and SY5 and less closely related to those from other gram negative biphenyl and chlorobiphenyl degraders, such as KF707 [53], LB400 [21], and DJ-12 [27]. They were also distantly related to genes encoding alkylbenzene degradation found in strains such as F1, RE204, IP01, and JR1, which indicates that the *bph* genes encoding the degradation of both alkylbenzenes and biphenyl from strain NCIMB 10643 had the same lineage

of molecular evolution as the genes encoding biphenyl degradation rather than the genes identified in the alkylbenzene degradation pathways. In addition, the results of both current study and the previous study carried out by Smith and Ratledge [48] demonstrate that the *bph*-encoded enzymes from strain NCIMB 10643 were most active towards substrates derived from biphenyl.

The *bph* genes in strain A5 are known to be clustered in the following configuration, *bphSEGF(orf4)A1A2A3BCD (orf1)A4R* [32, 50] (Fig. 4). The gene products then catalyze the conversion of biphenyl/PCB into acetyl-CoA, pyruvate, and a (chloro)benzoate. The *bph* cluster spanning ca 13 kb is located in the middle of a 55 kb catabolic transposon, Tn*4371* [32]. The PCR analysis (Fig. 4) carried out in the current study also showed that the *bph* genes in strain NCIMB 10643 and Tn*4371* were closely related, thereby suggesting that the *bph* genes of strain NCIMB 10643 could be on the same transposon, Tn*4371*. The present study also revealed overlapping sets of growth substrates that were degraded by the two degradation pathways. A recent study on the DNA-DNA hybridization of PCB-degrading strains isolated from widely distant polluted sites showed that the *bph* genes present in Tn*4371* were highly conserved in different (chloro)biphenyl-degrading hosts belonging to β-Proteobacteria such as *Acidovorax*, *Comamonas*, *Burkholderia*, *Achromobacter*, and *Alcaligenes* [50]. This indicates that *bph* genes similar to those found in Tn*4371* are widespread in nature and may also be involved in the degradation of alkylbenzenes.

One additional interesting finding from the current study was the presence of an OmpW family protein, Orf1, in the degradation pathway of strain NCIMB 10643. As mentioned in the Results section, members of this family of proteins are found encoded in the operons that degrade polycyclic aromatic hydrocarbons and alkanes in Gram-negative bacteria. A structural analysis of Omp21, an OmpW-type protein found in *C. acidovorans*, suggested that it contained 8 amphipathic β-strands forming a β-barrel that resembled 16- and 18-stranded porins [2, 3]. No clear evidence has yet been provided on the role of OmpW family proteins in the aromatic hydrocarbon and alkane degradation pathways. Accordingly, further studies are currently being carried out to clarify the biochemical role of the outer membrane proteins that have been found in the degradation pathways.

Acknowledgements

This work was supported by a Korea Research Foundation Grant (KRF 200-005-D20020). We would like to thank Drs. D. T. Gibson, R. E. Parales, S. E. Lindow, and S. -C. Kho for providing a strain or plasmid, and Dr. R. E. Parales for his suggestions to improve the quality of this paper.

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