Polychlorinated Biphenyl/Biphenyl Degrading Gene Clusters in *Rhodococcus* sp. K37, HA99, and TA431 Are Different from Well-Known *bph* Gene Clusters of Rhodococci

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Received October 5, 2006; Accepted February 19, 2007; Online Publication, May 7, 2007

[doi:10.1271/bbb.60551]

Four kinds of polychlorinated biphenyl (PCB)-degrading *Rhodococcus* sp. (TA421, TA431, HA99, and K37) have been isolated from termite ecosystem and under alkaline condition. The *bph* gene cluster involved in the degradation of PCB/biphenyl has been analyzed in strain TA421. This gene cluster was highly homologous to *bph* gene clusters in *R. globerulus* P6 and *Rhodococcus* sp. RHA1. In this study, we cloned and analyzed the *bph* gene cluster essential to PCB/biphenyl degradation from *R. rhodochrous* K37. The order of the genes and the sequence were different in K37 than in P6, RHA1, and TA421. The *bphC8*<sub>K37</sub> gene was more homologous to the meta-cleavage enzyme involved in phenanthrene metabolism than *bphC* genes involved in biphenyl metabolism. Two other *Rhodococcus* strains (HA99 and TA431) had PCB/biphenyl degradation gene clusters similar to that in K37. These findings suggest that these *bph* gene clusters evolved separately from the well-known *bph* gene clusters of PCB/biphenyl degraders.

**Key words:** *bph* gene cluster; biphenyl; *Rhodococcus*; biodegradation

Polychlorinated biphenyls (PCBs) are artificial biphenyl compounds containing 1 to 10 chlorine atoms. They are global persistent pollutants that disrupt endocrine function. PCB/biphenyl-degrading bacteria have been isolated from environments and can cometabolize many PCB congeners to chlorobenzoate through either oxidative or reductive routes.

The initial oxidative degradation of PCBs or biphenyls in aerobic bacteria consists of four steps, and yields chlorobenzoate or benzoate and 2-hydroxypenta-2,4-dienoate via biphenyl 2,3-dihydrodiol, 2,3-dihydroxybiphenyl (23DHBP), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). Four enzymes, biphenyl dioxygenase (BphA), dihydrodiol dehydrogenase (BphB), 2,3-dihydroxybiphenyl dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD), catalyze these steps.1) BphA consists of large (BphA1) and small (BphA2) terminal dioxygenase subunits, a ferredoxin (BphA3), and a ferredoxin reductase (BphA4). Usually, the genes for the *bphA*, *bphB*, and *bphC* genes are clustered and transcribed as an operon.2–5) One of the degradation products, 2-hydroxypenta-2,4-dienoate, is further metabolized to pyruvate and acetyl-CoA by successive action of 2-hydroxypenta-2,4-dienoate hydratase (BphE), 4-hydroxy-2-oxovalerate aldolase (BphF), and acetaldehyde dehydrogenase (BphG).

*Rhodococcus* is a Gram-positive bacterium belonging to the nocardioform actinomycetes.6,7) Four kinds of *Rhodococcus* strains that degrade PCB/biphenyl have been isolated from the termite ecosystem (*R. erythropolis* TA421 and *R. erythropolis* TA431), or under alkaline conditions (*R. rhodochrous* K37 and *Rhodococcus* sp. HA99).8,9) Some *Rhodococcus* strains have multiple extradiol dioxygenase genes. TA421 has seven *bph* genes (*bphC1* to *bphC7*).10,11) In addition, TA421 carries an approximately 500-kb linear plasmid that contains three of the seven *bph* genes (*bphC2*, *bphC3*, and *bphC4*).12) Mutant strain TA422, which spontaneously loses the plasmid, dose not grow on biphenyl. In strain TA421, the *bphC3* and *bphC4* genes are accompanied by genes encoding the enzymes needed for biphenyl degradation (*bphA* and *bphB*), and it appears that gene products of *bphC3* and *bphC4* are involved in biphenyl metabolism.11,12)

In contrast, *Rhodococcus* sp. RHA1 and *R. globerulus* P6 have six (*bphC1* to *bphC5* and *etbC*) and three...
New Type bph Gene Clusters in Rhodococcus sp. 1137

(bphC1 to bphC3)² bphC genes respectively. Three genes (bphC1, bphC5, and etbC) are induced by biphenyl and ethylbenzene, suggesting that they are involved in biphenyl degradation in RHA1. The bphC1 gene of P6 and the bphC gene of RHA1, which are necessary for biphenyl degradation, are closely related to bphC3 of TA421.

Southern hybridization analysis of total DNA from TA431, K37, HA99, and P6 using the seven bphC genes from TA421 as probes² showed that TA431, K37, and HA99 lack genes corresponding to bphC3 and bphC4 in TA421; however, these three bacteria can utilize biphenyl and cometabolize PCBs. It has been suggested that PCB/biphenyl degradation gene clusters in TA431, K37, and HA99 are different from the well-known bph gene clusters in P6, RHA1, and TA421.

We have reported that K37 has eight kinds of extradiol dioxygenase genes (bphC1K37 to bphC8K37), and that the bphC6K37 and bphC7K37 genes are located on a 200-kb linear plasmid. Furthermore, only the bphC8K37 gene was induced by biphenyl, and the Km value of the 2,3-dihydroxybiphenyl dioxygenase activity for the BphC8K37 product was much lower for the other BphC K37 products.¹⁴

In this study, we confirmed that the bphC8K37 gene is essential for degradation of biphenyl in K37 and analyzed the bph gene cluster. We found that the order of bph genes was different from those in other Rhodococci. The identity of the amino acid sequence of the bph genes was also low. In addition, we cloned the homologous genes from TA431 and HA99.

Methods and Materials

Bacterial strains and culture conditions. Rhodococcus species (strains K37, HA99, TA421, and TA431) were grown in LB or C-medium (pH 7.0)⁹ containing biphenyl, aromatic compounds, or steroids at 0.1 or 0.5% final concentration at 30°C.

Cloning and sequence analysis of the regions upstream and downstream of the bphC8K37 gene from R. rhodochrous K37. Rhodococcus strains were treated with 20 mg/ml lysozyme for 2 h at 37°C, and total DNA was prepared according to the method of Sambrook et al.¹⁵ A 3.1-kb ApaI fragment including the bphC8K37 of K37 has been cloned (pKAC8).¹⁴ The regions upstream and downstream of bphC8K37 were cloned in pBluescript SK+ or SK+. A 3.9-kb EcoRI fragment downstream of pKAC8 was cloned (pKC8D1) using a 400-bp ApaI/BstXI fragment containing the 3′-end of pKAC8 as a probe. Furthermore, additional downstream regions, a 5.9-kb ApaI and a 7.5-kb ClaI fragment, were cloned using fragments containing each 3′-terminal region as a probe (pKC8D2 and pKC8D3). A 950-bp ApaI/SphI fragment was used as a probe to clone the upstream region of bphC8K37. A 6.5-kb PstI fragment upstream of bphC8K37 was cloned (pK8U1). The appropriate DNA fragments were subcloned into pBluescript KS+ and SK+ and deleted using a Kilo-Sequence Deletion Kit (Takara Bio, Ohtsu) for sequencing. Nucleotide sequencing was accomplished by the dideoxynucleotide chain termination method with a model 377 sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed by GENETYX-MAC (Software Development, Tokyo).

Isolation of non-biphenyl-degrading R. rhodochrous K37 mutant strains by homologous recombination and spontaneous loss of the linear plasmid. The 1.5-kb kanamycin resistance gene was inserted into the NeoI site in bphC8K37 within plasmid pKAC8, which contains a 3.1-kb ApaI fragment of the K37 linear plasmid. The resulting plasmid was digested with ApaI. The released 4.6-kb fragment containing the bphC8K37 and kanamycin resistance genes was transferred into K37 cells by electroporation. The electroporation conditions for introduction of DNA with Gene Pulser (Bio-Rad Laboratories, Richmond, CA) and a 2-mm gapped cuvette were as follows: capacitance, 25 μF; parallel resistors, 800 Ω; and initial voltage, 2.0 kV. Strains that were kanamycin-resistant and unable to grow on biphenyl were selected. Replacement of the disrupted bphC8K37 was confirmed by Southern hybridization using a labeled 1.5-kb SphI fragment as the bphC8K37 probe.

In the same way, the bphA1K37 disruptant was constructed. The kanamycin resistance gene was inserted into the BstPI site in bphA1K37 within plasmid pKAC8D1. A 5.5-kb EcoRI fragment containing bphA1K37 with kanamycin resistance genes was released and transferred into K37 cells. Strains that were kanamycin-resistant and unable to grow on biphenyl were selected. Southern hybridization was done using the labeled 400-bp BstXI-ApaI fragment as a probe.

A mutant that spontaneously lost the ability to grow on biphenyl was obtained as follows: Strain K37, which is usually grown at 30°C, was grown at 37°C in LB. Appropriate dilutions of the culture were spread onto LB plates. Colonies were transferred to an LB plate or a C-medium plate containing biphenyl as sole carbon source. Cells that grew on LB but not on C-medium containing biphenyl were selected.

Pulsed-field gel electrophoresis (PFGE) and Southern hybridization. DNA samples for PFGE and Southern hybridization were prepared as described by McClelland et al., with the following modifications: K37, TA431, and HA99 cells were grown in LB medium. The cells were treated with 2 mg/ml lysozyme in SPE buffer (10% sucrose, 10 mM EDTA, and 10 mM sodium phosphate, pH 7.0) before they were embedded in agarose. Electrophoresis was performed using a CHEF Mapper system (Bio-Rad) with a 1% agarose gel in 0.5× TBE buffer (10× TBE consisted of 0.89 mM Tris, 0.89 mM borate, and 22 mM EDTA) at 14°C. After PFGE, the gel was blotted onto a nylon membrane, and Southern hybrid-
ization was performed with the bphC8K37 probe at 42 °C by the DIG system (Boehringer Mannheim Yamanouchi, Tokyo).

Partial purification of 2,3-dihydroxybiphenyl dioxygenase from R. rhodochrous K37 grown on biphenyl. K37 cells grown on biphenyl as sole carbon source were suspended in 10 mM potassium–phosphate buffer, pH 7.5, containing 1 mM dithiothreitol, and disrupted with a French press. Cell debris was removed by centrifugation. The supernatant was passed through a DEAE Toyopearl-650M (Tosoh, Tokyo) equilibrated with the same buffer. 2,3-Dihydroxybiphenyl dioxygenase was eluted with a buffer including 250 to 300 mM NaCl. This solution was passed through gel filtration on Superdex 200 HR 10/30 with fast protein liquid chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ). The active fraction was concentrated and applied to a Mono-Q HR 5/5 (Pharmacia LKB Biotechnology). The enzyme was eluted with a linear gradient of 0.2 to 0.6 M NaCl in 10 mM buffer. The fraction containing 2,3-dihydroxybiphenyl dioxygenase activity was concentrated. The partially purified enzyme was separated by electrophoresis on a nondenaturing 10% acrylamide gel. After electrophoresis, the gel was equilibrated in 50 mM Tris–HCl buffer (pH 7.5) for 10 min, transferred to 50 mM Tris–HCl buffer containing 5 mM 23DHBP, and incubated for 5 min at room temperature to allow yellow meta-cleavage compounds to form in the presence of 2,3-dihydroxybiphenyl dioxygenase. The gel was transferred to a polyvinylidene difluoride membrane. The N-terminal amino acid sequence was determined by the 492 Procise Protein Sequencing System (Applied Biosystems). Enzyme activity was measured using 200 μM 23DHBP as the substrate in 50 mM Tris–HCl (pH 7.5).

Cloning of bphC8K37 gene homologs from Rhodococcus sp. HA99 and R. erythropolis TA431. Total DNA from strains HA99 and TA431 was digested with several restriction enzymes. After agarose gel electrophoresis, the gel was blotted onto a nylon membrane, and Southern hybridization was performed using a DIG-labeled bphC8K37 probe, which consisted of the 1.5-kb SphI fragment of pKAC8. An approximately 8.0-kb EcoRV DNA fragment of HA99 and an approximately 8.2-kb BamHI DNA fragment of TA431 were hybridized with the bphC8K37 probe. These fragments were separated by agarose gel electrophoresis and cloned into pBlueScript KS+. Positive clones were selected by Southern hybridization with the bphC8K37 probe. The appropriate DNA fragments were subcloned into pBlueScript KS+ and SK+ and deleted using the Kilo-Sequence Deletion Kit (Takara Bio) for sequencing. Northern hybridization was done according to the previously reported method.14) Electrophoretically purified PCR products of bphA1K37 were used in the preparation of DIG-labeled probe.

Expression bph genes in R. rhodochrous K373 and HPLC analysis. A BamHI 9.6 kb fragment containing bphB3K37, bphC3K37, bphA1K37, bphA2K37, bphA3K37, and bphA4K37 was cloned from K37 in E. coli with pBlue-script KS+. A fragment digested with BamHI was blunted and introduced into the blunted KpnI site of E. coli-Rhodococcus shuttle vector pKK-4017 (designated pKBCA). Constructed plasmids were introduced into K373 by electroporation.

K373 cells harboring pKBCA grown on LB were washed twice with 10% B-solution (10 mM phosphate buffer, pH 7.0) and resuspended in 10% B-solution, adjusted for optical density at 600 nm of approximately 2.5, and then used for biphenyl conversion. The cells were incubated with 0.1% biphenyl at 30 °C. Supernatants after centrifugation were injected into high-performance liquid chromatography (HPLC). HPLC analysis was performed according to a modified method, as described previously,10) followed by treatment with an acetonitrile/water/trifluoroacetic acid (50:50:0.05) solvent system. Meta-cleavage products used as a positive control for HPLC were prepared as follows: after E. coli harboring pKAC8 containing the bphC8K37 was cultivated, 23DHBP was added as substrate.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB-272984 (K37), AB272985 (TA431), and AB272986 (HA99).

Results

Isolation of non-biphenyl-degrading mutant strains of R. rhodochrous K37

It has been reported that the linear plasmid of TA421 is spontaneously lost at a frequency of 2% when TA421 is grown on LB medium at 30 °C.12) This mutant cannot grow on biphenyl as the sole carbon source. Mutant strains that cannot grow on biphenyl were not obtained from K37 when it was grown on LB at 30 °C; however, mutant strains of K37 lacking the ability to grow on biphenyl as sole carbon and energy source were isolated when the bacteria were grown at 37 °C. Three non-biphenyl-degrading mutant strains were obtained from approximately 1,000 screened colonies. We designated these mutant strains K371, K372, and K373. Except for their inability to grow on biphenyl, their ability to grow did not differ from the wild type, such as benzoate, phenol, naphthalene, or testosterone.

To determine the genetic differences between the eight bphC genes of K37 non-biphenyl-degrading strains K371, K372, and K373, we performed Southern hybridization analysis of total DNA from the three strains. We found that only bphC8K37 was lost from strain K371, but that both bphC6K37 and bphC8K37 were lost in strains K372 and K373 (Table 1).
The \textit{bphC6}_{K37} and \textit{bphC8}_{K37} genes are known to be located on a linear plasmid in strain K37. To investigate the size of the large linear plasmid in these mutant strains, we performed PFGE on undigested total DNA from strains K371, K372, and K373. PFGE revealed that the 200-kb plasmid was entirely lost from strains K372 and K373 (Fig. 1). On the other hand, a new approximately 160-kb band replaced the 200-kb band in strain K371. This 160-kb plasmid was formed from the 200-kb plasmid by a loss of the region that includes \textit{bphC8}_{K37}, because \textit{bphC6}_{K37} was present on the 160-kb plasmid (Table 1). These results indicate that the approximately 40-kb region containing the \textit{bphC8}_{K37} was necessary for growth on biphenyl.

\textit{N}-Terminal amino acid sequences of the \textit{meta}-cleavage enzyme induced by biphenyl in \textit{R. rhodochrous} K37

Strain K37 was grown in C-medium containing biphenyl as sole carbon and energy source. An extradiol dioxygenase was partially purified by sequential column chromatography. The partially purified protein was separated by 10% nondenaturing polyacrylamide gel electrophoresis (PAGE). After PAGE, the extradiol dioxygenase activity in the gel was visualized by soaking it in 23DHBP. A single bright yellow band, due to formation of the \textit{meta}-cleavage product of 23DHBP, appeared in the gel. This band was transferred to a polyvinylidene difluoride membrane, and its \textit{N}-terminal amino acid sequence was determined to be NH\textsubscript{2}-MVKSLAYMGVTSPALAE, which is the same as the deduced amino acid sequence of \textit{BphC8}_{K37} in strain K37.\textsuperscript{14)}

\textbf{Isolation and analysis of a bph gene cluster from \textit{R. rhodochrous} K37}

A 3.0-kb \textit{ApaI}-digested fragment including \textit{bphC8}_{K37} has been cloned (pKAC8).\textsuperscript{14)} In addition, we cloned the regions upstream and downstream of pKAC8 and generated a physical and genetic map (Fig. 2). This region had a nucleotide sequence of 16,340-bp and 12 open reading frames (ORFs), including \textit{bphC8}_{K37}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Probes} & \textbf{Hybridization with} & \textbf{Genomic} & \textbf{location} \\
 & \textbf{the total DNA of} & & \\
\textbf{K371} & \textbf{K372} & \textbf{K373} & \\
\hline
\textit{bphC1} & + & + & + & Chromosome \\
\textit{bphC2} & + & + & + & Chromosome \\
\textit{bphC3} & + & + & + & Chromosome \\
\textit{bphC4} & + & + & + & Chromosome \\
\textit{bphC5} & + & + & + & Chromosome \\
\textit{bphC6} & + & − & − & 200 kb Linear plasmid \\
\textit{bphC7} & + & + & + & Chromosome \\
\textit{bphC8} & − & − & − & 200 kb Linear plasmid \\
\hline
\end{tabular}
\caption{Genetic Differences between the Eight \textit{bphC} Genes of K37 and Non-Biphenyl-Degrading Strains}
\end{table}

\textsuperscript{14} Isolation and analysis of a bph gene cluster from \textit{R. rhodochrous} K37

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\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{PFGE of Total DNA from Wild-Type \textit{R. rhodochrous} Strain K37 and Non-Biphenyl Degrading \textit{R. rhodochrous} Strains K371, K372, and K373. Lane 1, Total DNA from wild-type \textit{R. rhodochrous} K37; lane 2, total DNA from \textit{R. rhodochrous} K371; lane 3, total DNA from \textit{R. rhodochrous} K372; lane 4, total DNA from \textit{R. rhodochrous} K373. The molecular sizes were estimated by comparison with a \textit{Saccharomyces cerevisiae} chromosome standard marker set.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Genetic Map of the \textit{bph} Gene Cluster for PCB/Biphenyl Degradation in \textit{R. rhodochrous} K37. The open bar indicates the sequenced region. Solid bars indicate clones. Restriction sites where the kanamycin resistance gene was inserted are indicated by small white arrowheads. Arrows with solid lines indicate the positions and directions of the \textit{bph} genes and their ORFs. Abbreviations for restriction enzymes: N, \textit{NotI}; A, \textit{ApaI}; B, \textit{BamHI}; E, \textit{EcoRI}; P, \textit{PstI}; C, \textit{ClaI}.}
\end{figure}

\textbf{Table 1. Genetic Differences between the Eight \textit{bphC} Genes of K37 and Non-Biphenyl-Degrading Strains}

\textsuperscript{1} Genes \textit{bphC1} to \textit{bphC8} of K37 were used individually.

\textsuperscript{2} −, Probe did not hybridize. +, Probe hybridized.
been cloned and analyzed. The order of bph genes in the clusters from these three strains was similar, and the amino acid sequences were highly homologous (Fig. 3), but the order and amino acid sequence homology in the bph gene cluster of strain K37 was considerably different. BphC8K37 had 61.7% amino acid sequence identity with PhdF from Nocardoides sp. KP7, whereas the other known BphC had a sequence identity below 41%. PhdF is an extradiol dioxygenase participating in the degradation of phenanthrene in Nocardoides sp. KP7. Also, BphB K37 and BphA4K37 had the highest amino acid sequence identities with PhdE, a dihydrodiol dehydrogenase (62.7%), and PhdD, a ferredoxin reductase (44.2%), of Nocardoides sp. KP7. There were no homologous proteins with an identity higher than 40% with BphA1K37, BphA2K37, BphA3K37, or BphD K37 (Fig. 3).

Comparison of the order of the bph gene cluster for biphenyl degradation by strain K37 and the phd gene cluster for phenanthrene degradation by Nocardoides sp. KP7 showed that the first five genes (bphB K37, bphC8K37, orf82K37, bphA1K37, and bphA2K37) of K37, and phdE, phdF, orf131, phdA, and phdB of Nocardoides sp. KP7) had the same order, but the region immediately upstream of bphB K37 and the downstream region of orf85K37 were quite different (Fig. 3). The phdA and phdB genes encode the α and β respectively subunits of phenanthrene dioxygenase, and correspond to bphA1 and bphA2 of biphenyl dioxygenase. The hypothetical transposase and helper protein genes (orf810K37 and orf811K37), which are similar to those of the IS21 family, were found downstream of the bph gene cluster in K37. An IS110 transposase-like gene fragment was also found upstream of the bph gene cluster. The functions of orf82K37, orf85K37, and orf86K37 were not clear because there were no homologous genes in the database.

We found that expression of bphC8K37 was induced into the C-medium containing biphenyl in strain K37. To determine whether bphA1K37 is induced in biphenyl containing medium, Northern hybridization was performed using the PCR amplified bphA1K37 gene as a probe. Total RNA was prepared from K37 cells after a 5-h incubation on a medium containing biphenyl, succinate, naphthalene, fluorene, benzoxae, phenol, cumene, phenanthrene, testosterone, cabazole, or dibenzofuran. Consistently with the results for bphC8K37, the probe hybridized only with RNA isolated from biphenyl grown cells (data not shown). These results suggest that these bph genes construct the operon.

Disruption of the bphC8K37 and bphA1K37 genes of R. rhodochrous K37

To examine whether the bphC8K37 and the bphA1K37 genes are essential to biphenyl degradation in K37, we replaced them with disrupted versions through homologous recombination. The 1.5-kb kanamycin resistance gene was inserted into the NcoI site within bphC8K37. Transformation of K37 was carried out using the 4.5-kb ApoI fragment harboring bphC8K37 introducing a kanamycin resistance gene into the NcoI site. We isolated two mutant strains that can grow on testosterone but not on biphenyl. Based on Southern hybridization analysis, in two of the bphC8K37 disruptants, bphC8K37 was precisely replaced with the kanamycin resistance gene-inserted bphC8K37. These two disruptants did not grow in C-medium containing biphenyl and kanamycin. Otherwise, they grew as well as the wild type on several other carbon sources. In the same way, the kanamycin resistance gene was inserted into the BstPI site within bphA1K37. A disruptant, precisely replaced with the kanamycin resistance gene-inserted bphA1K37, was isolated. It grew on LB medium and on C-medium containing testosterone, but not on biphenyl. These
Biphenyl conversion by expressed bph genes in R. rhodochrous K373

Biphenyl conversion was not detected in E. coli harboring the 9.6-kb BamHI fragment encoding the bphB to bphA4 genes as a result of HPLC analysis, although BphC activity was detected. Hence we attempted to express these genes in the Rhodococcus strain. The 9.6-kb BamHI fragment was introduced into E. coli-Rhodococcus shuttle vector pRK401 (designated pKBCA) (Fig. 2). The construct was introduced into K373, which had spontaneously lost the 200-kb linear plasmid containing the bph gene cluster from K37. At the same time, we transformed K373 with shuttle vector pRK401 to obtain a negative control. A 434 nm peak, consistent with that of HOPDA, was found in the absorption spectrum of supernatants of a 10%-B solution with biphenyl from K373 harboring pKBCA (data not shown). Furthermore, supernatants were analyzed by HPLC. 23DHBP was added to a 10%-B solution with biphenyl 2,3-dihydrodiol having a 304 nm absorption spectrum. In contrast, a specific peak corresponding to biphenyl 2,3-dihydrodiol was not obtained for the negative control culture (Fig. 4C). The vertical axis indicates wavelength, the horizontal axis indicates retention time, and absorbance of each compound is represented by a contour line.

Analysis of bphC8K37 homologs from R. erythropolis TA431 and Rhodococcus sp. HA99

We carried out Southern hybridization analysis on total DNA from TA431, HA99, TA421, and P6 using the bphC8K37 gene as a probe. TA431 and HA99 have genes corresponding to bphC8K37, but TA421 and P6 do not. We cloned and analyzed an 8.0-kb EcoRV DNA fragment from HA99 and an 8.2-kb BamHI DNA fragment from TA431 using bphC8K37 as a probe. Combined with analysis of upstream and downstream nucleotide sequences, a total of 11,772 bp from HA99 and 11,200 bp from TA431 were sequenced for these bphC genes (Fig. 5A).

Gene TA431 was 99.7% identical with the K37 gene over 8,529 bp, including the bph gene cluster, except that the TA431 gene had a 15-bp repeated sequence in the bphA3TA431. Over a 10,025-bp stretch, the HA99 gene was 99.9% identical with the same region in K37, but it was evident that the transposase and the helper protein genes of the IS21 family downstream of the bph gene cluster were inserted in the CGGGG sequence of strain K37 because the CGGGG sequence was duplicated upstream and downstream of orf810K37 and orf811K37 (Fig. 5B). A DNA invertase-like gene and defective transposase- and transporter-like genes were found upstream of the bph gene cluster in HA99. The ORFs located downstream of the bph gene cluster from TA431 (ORF1TA431 to ORF5TA431) had homology with ORF1 to ORF5, located downstream of the naphthalene degradation gene in Rhodococcus sp. NCIMB12038. The amino acid sequences of BphA1HA99, BphA2HA99, BphA3HA99, and BphDHA99 from HA99, and BphA3TA431 and BphDTA431 from TA431, were 100% identical to those from K37. Other Bph products of HA99 and TA431 had amino acid sequence identities greater than 99% with those of K37.

It has been reported that bphC8K37 is located on a 200-kb linear plasmid. To determine whether the bph gene clusters of HA99 and TA431 are located on a linear plasmid, we performed PFGE and subsequent Southern hybridization analysis using bphC8K37 as a probe. Strain HA99 had a 270-kb linear plasmid, and TA431 had a
570-kb linear plasmid. Southern hybridization showed that the probes generated from the \textit{bphC8} \textit{K37} homologs of HA99 and TA431 hybridized with the linear plasmids (Fig. 6). These results indicate that the \textit{bph} gene clusters of HA99 and TA431 are also located on linear plasmids.

To determine whether the \textit{bphC8} \textit{K37} and the \textit{bphA1} \textit{K37} homologs of HA99 and TA431 are in fact induced when these strains are grown on biphenyl, we carried out Northern hybridization analysis. HA99 and TA431 were grown on C-medium containing biphenyl, succinate, naphthalene, phenanthrene, phenol, benzoate, cumene, or testosterone. Northern hybridization analysis was performed using the \textit{bphC8} \textit{K37} or \textit{bphA1} \textit{K37} probe. Each probe was hybridized with RNA isolated from cells grown only on biphenyl (data not shown). These results suggest that the \textit{bphC8} \textit{K37} and \textit{bphA1} \textit{K37} homologs of HA99 and TA431 are similarly controlled, and that these genes also constructed an operon.

\section*{Discussion}

\textit{bph} gene clusters that have high homology with genes from TA421 have been analyzed in \textit{Rhodococcus} sp. strains P6 and RHA1.\textsuperscript{2,4} The nucleotide sequences of \textit{bphC1} from P6 and \textit{bphC} of RHA1, which participate in the degradation of biphenyl, were 99\% and 71\% identical to \textit{bphC3} from TA421, respectively. Southern hybridization analysis indicates that genes corresponding to \textit{bphC3} and \textit{bphC4} of TA421 are absent from strains K37, HA99, and TA431.\textsuperscript{12} It is expected that these strains have other types of \textit{bph} gene cluster that participate in the degradation of PCBs and biphenyls.

Here we examined the full length of the \textit{bph} gene cluster from K37. We found that the order and sequences of the genes in strain K37 were clearly different from the highly homologous \textit{bph} genes in the clusters of other reported \textit{Rhodococcus} strains. According to a homology search in a DNA data base, \textit{BphC8} \textit{K37} has
higher amino acid sequence identity with the meta-cleavage enzyme involved in phenanthrene degradation (PhdF) from Nocardioides sp. K719 than with the meta-cleavage enzyme involved in biphenyl degradation. The bphB\textsubscript{K37}, bphD\textsubscript{K37}, orf\textsubscript{85\textsubscript{K37}}, and orf\textsubscript{85\textsubscript{K37}} genes also have the highest identity with the corresponding genes of Nocardioides sp. K71. A comparison of the bphB\textsubscript{K37}, bphC\textsubscript{K37}, and bpha\textsubscript{1\textsubscript{K37}} genes of K37 with the phdE, phdF, and phdA genes of Nocardioides sp. K71 revealed that they were highly homologous and had similar lengths. This suggests that the regions of the bph gene cluster share an ancestor with the region of the phd gene cluster, and that the gene clusters evolved to function in each metabolic system.

Nocardioides sp. K71 does not grow on naphthalene or biphenyl, but it does grow on phenanthrene.\textsuperscript{9,10} This organism degrades phenanthrene via 1-hydroxy-2-naphthoate, ortho-phthalate, and protocatechuate.\textsuperscript{21–23} Its phd gene cluster is localized on the chromosome, and the order of the phd genes is quite different from that of other reported analogous gene sets for polycyclic aromatic hydrocarbon degradation.\textsuperscript{24–26} The phenanthrene dioxygenase (phdABCD) genes of Nocardioides sp. K71 induced in E. coli do not transform monomeric aromatic hydrocarbons including biphenyl, but they do convert tricyclic fused aromatic hydrocarbons such as phenanthrene, anthracene, and fluorene.\textsuperscript{27} In contrast to Nocardioides sp. K71, K37 grows on naphthalene but not on phenanthrene. HPLC analysis revealed that when phenanthrene was added to K37 grown on biphenyl, a transformed compound was formed (data not shown). It is possible that phenanthrene was converted by enzymes coding in the bph gene cluster.

Except for PhdF, BphC\textsubscript{K37} has low homology with other reported extradiol dioxygenases (≤ 41%). Phylogenetic analysis indicates that BphC\textsubscript{K37} is part of a new subfamily involved in biphenyl degradation.\textsuperscript{14} We identified the bphC genes that had high identities with bphC\textsubscript{K37} in strains HA99 and TA431, and then analyzed the bph gene clusters, including the bphC\textsubscript{K37} homolog. These bph gene clusters in K37 had more than 99% nucleotide sequence identities with the corresponding 8529- and 10,025-bp regions in TA431 and HA99 respectively (Fig. 5A). Because these bphC genes were induced by biphenyl, it appears that their gene products also participate in the degradation of PCB/biphenyl in strains HA99 and TA431. Furthermore, in strains K37, HA99, and TA431, these bph gene clusters are present on the linear plasmid (Fig. 6), and there are some signs of recombination around these bph gene clusters, such as insertion of transposase or DNA invertase sequences. These results suggest that these bph gene clusters evolved separately from the clusters of other known Rhodococcus strains and were transferred quite recently, and that the bph gene clusters were spread in Rhodococcus by horizontal transfer.

We have found that strain K37 has eight bphC genes, that the bphC\textsubscript{8\textsubscript{K37}} gene is located on a 200-kb linear plasmid, and that of the eight bphC genes, only bphC\textsubscript{8\textsubscript{K37}} is induced by biphenyl.\textsuperscript{14} In this study, we found that the active enzyme corresponding to the bphC\textsubscript{8\textsubscript{K37}} gene product was expressed by K37 grown on biphenyl, and that mutant strains that lost the 200-kb linear plasmid entirely, or 40-kb including bphC\textsubscript{8\textsubscript{K37}}, and had a disrupted bphC\textsubscript{8\textsubscript{K37}} gene did not grow on biphenyl. These results indicate that bphC\textsubscript{8\textsubscript{K37}} is essential for the growth of strain K37 on biphenyl, and that the transcription of each bphC gene is strictly regulated. Similarly, expression of bpha\textsubscript{1\textsubscript{K37}} was also detected only when biphenyl was used as a carbon source and the bpha\textsubscript{1\textsubscript{K37}} disrupted mutant did not grow on biphenyl. This suggests that bpha\textsubscript{1\textsubscript{K37}} is also a component of the degradation pathway of biphenyl. Furthermore, strain K373 introduced into the region bphB\textsubscript{K37} to bpha\textsubscript{4\textsubscript{K37}} transformed biphenyl to HOPDA, whereas strain K373, harboring shuttle vector pRK401 did not convert biphenyl. These results strongly suggest that expressed Bpha\textsubscript{1\textsubscript{K37}}, Bpha\textsubscript{2\textsubscript{K37}}, Bpha\textsubscript{3\textsubscript{K37}}, Bpha\textsubscript{4\textsubscript{K37}}, BphB\textsubscript{K37}, and BphC\textsubscript{K37} have an activity of biphenyl transformation. The results of the present and previous studies indicate that the cloned bph gene cluster is essential for PCB/biphenyl degradation in K37.

Rhodococci are thought to play an important role in the biodegradation of environmental pollutants in soils due to their ability to transform a wide range of xenobiotic compounds, including PCBs.\textsuperscript{28–30} It is thought that combining various genes enables them to degrade a variety of compounds.

Acknowledgment

This work was partly supported by grants from the Ecomolecular Science Research Program of RIKEN.

References

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