BphS, a Key Transcriptional Regulator of bph Genes Involved in Polychlorinated Biphenyl/Biphenyl Degradation in Pseudomonas sp. KKS102*

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The bph genes in Pseudomonas sp. KKS102, which are involved in the degradation of polychlorinated biphenyl/biphenyl, are induced in the presence of biphenyl. In this study our goal was to understand the regulatory mechanisms involved in the inducible expression. The bph genes (bphEGForf4)A1A2A3BCD(orfl)A4R constitute an operon, and its expression is strongly dependent on the pE promoter located upstream of the bphE gene. A bphS gene, whose deduced amino acid sequence showed homology with the GntR family transcriptional repressors, was identified at the upstream region of the bphE gene. Disruption of the bphS gene resulted in constitutive expression of bph genes, suggesting that the bphS gene product negatively regulated the pE promoter. The gel retardation and DNase footprinting analyses demonstrated specific binding of BphS to the pE promoter region and identified four BphS binding sites that were located within and immediately downstream of the −10 box of the pE promoter. The four binding sites were functional in repression because their respective elimination resulted in derepression of the pE promoter. The binding of BphS was abolished in the presence of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, an intermediate compound in the biphenyl degradation pathway. We concluded that the negative regulator BphS plays a central role in the regulation of bph gene expression through its action at the pE promoter.

Human activities have created toxic compounds that cause environmental pollution and threaten the earth’s biosphere. Among these compounds, PCB is one of the most serious pollutants, and the microorganisms capable of degrading PCB have been studied worldwide (1, 2). Pseudomonas sp. KKS102 has been isolated (3) and shown to degrade PCB/biphenyl via a meta cleavage pathway to yield a tricarboxylic acid cycle intermediate and benzoic acid (4–7) (see Fig. 1A). The genes coding enzymes for this conversion have been sequenced, characterized, and shown to be clustered as bphEGForf4A1A2A3BCD(orfl)A4R (see Fig. 1B).

In many microorganisms capable of degrading chemical compounds, the transcription of genes involved in the degradation is regulated (8). In most cases, the genes coding for the regulator exist near the structural genes, and their protein products activate the transcription in the presence of their cognate inducer molecule. Repressor-mediated regulation is rare for genes involved in the catabolism of aromatic compounds. However, Mouz et al. (9) reported that the expression of bph genes on transposon Tn4371 was repressed by the product of bphS gene, although the molecular events in the repression and derepression have fully remained to be elucidated.

The bph genes and their organization in KKS102 are highly homologous to the bph genes on transposon Tn4371 (10, 11). These two bph gene clusters share 94% identity at the nucleotide level, but DNA sequences in the upstream region of bphE are different from each other (10).

The bph genes in KKS102 are induced when grown in the presence of biphenyl. This induction requires an inducer molecule, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), an intermediate metabolite of the biphenyl degradation pathway (12). In this study, we identified a negative regulator of bph genes and revealed its function in the regulation of bph gene expression in KKS102. This report describes for the first time the detailed regulatory mechanism of PCB/biphenyl degradation genes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Pseudomonas sp. KKS102 (4) and its derivatives were cultivated in 1/3 L broth (0.33% tryptone, 0.16% yeast extract, 0.5% NaCl) at 30 °C. Escherichia coli cells were grown in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C. Antibiotics were used at a final concentration of 50 μg/ml for ampicillin and 25 μg/ml for kanamycin and chloramphenicol.

Construction of the bphS Disruptant—For the construction of a plasmid for bphS disruption, plasmid pKH1004 carrying a 1.2-kb HinclII fragment in the multicloning site of pUC19 was digested by XhoI, and a chloramphenicol resistance gene derived from pHS399 was inserted into the cleaved site. The resulting plasmid was linearized by BamHI and HindIII digestion and used for electroporation. The gene disruption was confirmed by Southern blot analysis. The Southern blot analysis was performed with an ECL gene detection system (Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

Construction of Strains for LacZ Reporter Assay—All of the fusion constructs of the modified upstream region of bphE and lacZ were integrated into the genome of KKS102. For systematic construction of...
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Plasmids for integration, plasmid pKLZ-A was constructed. pKLZ-A contains the following DNA fragments: kanamycin resistance gene derived from Tn5 as a marker for integration, a synthetic terminator sequence to prevent read-through of transcription, a multicloning site comprised of EcoRI, SmalI, and BamHI sites, and lacZ gene derived from E. coli. The fragments were inserted into randomly selected sites of the KKS102 genome. The modified promoter-lacZ fusions were constructed by inserting either duplex oligonucleotides or polymerase chain reaction-amplified DNA fragments into the multicloning site of pKLZ-A or of its derivative plasmid. The DNA sequences inserted are presented in Fig. 5.

Construction of the pE Promoter-deleted Mutant—For the deletion of the chromosomal pE promoter, plasmid pH966A was constructed. The E. coli plasmid pH966A carries the following DNA fragments in the cloning site of pHS399 (see Fig. 8): a bphE upstream region (−400 to −1284 relative to +1) is the start codon for bphE), a DNA fragment derived from pKLZ-A containing the kanamycin resistance gene and a terminator sequence, an upstream region of the lacZ gene derived from E. coli, a fragment derived from pHS399 (see Fig. 8), and a fragment derived from pKLZ-A containing the lacZ gene derived from E. coli. The plasmid pH966A was digested by HindIII and BamHI in a vector sequence and used for electroporation.

Electroporation—Each of the plasmids was linearized by an appropriate restriction enzyme, extracted with phenol/chloroform, ethanol-precipitated, dissolved in water, and introduced into KKS102 by electroporation. The cells from liquid culture were washed five times with chilled sterile water. The Gene Pulser (Bio-Rad) was used under the following conditions: 0.1-cm cuvette, 200 ohms, 25 microfarads, 1.8 kV, and a pulse time of 4.7–5.0 ms. A 1 ml aliquot of SOC medium (2% D-glucose, 0.5% yeast extract, 0.5% NaCl, 10 mM MgCl₂, and 20 mM glucose, pH 7.0) was added immediately after the electric pulse. The cells were incubated at 30 °C for 3 h prior to plating onto 1/3 L broth containing the appropriate antibiotics.

Northern Blot Analysis—The total RNA was isolated by the method described by Hopwood et al. (14). Hybridization and detection were performed by using digoxigenin-labeled DNA with a CSPD system (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The 1.0-kb HindII-Apal fragment, 1.2-kb SphI-SmalI fragment, and 0.9-kb EcoT22I-KpnI fragment were used to generate bphA1, bphC, and bphD probes, respectively (see Fig. 1). The size of the bphE encoding region was determined by the method of Maxam and Gilbert (16).

RESULTS

Nucleotide Sequence of the Upstream Region of bphE—The upstream region of bphE, the first gene of the bph gene cluster, was sequenced for about 3 kb. Two ORFs were found in this region in an orientation opposite that of the bph genes cluster (Fig. 1B). The start codon of the ORF proximal to bphE was 511 bp distant from that of bphE. Their nucleotide and deduced amino acid sequences are shown in Fig. 2. The deduced amino acid sequence of the ORF proximal to bphE showed homology to transposases of several transposons. These are IS1405 from Ralstonia solanacearum (DDBJ accession number AF167984), IS5 from Pseudomonas putida (DDBJ accession number AF052751), and ISPMS from Pseudomonas syringae (DDBJ accession number AB023075). Identities between these ORF and their transposable element are 74, 64, and 62%, respectively. Typical terminal 4-bp direct repeats and 16-bp inverted repeats flank this ORF. Because these are common features of an insertion sequence, we designated this region and the transposase ORF as ISBPH and invBPH, respectively. Southern blot analysis using a 1374-bp SphI fragment (Fig. 1B) as a probe revealed that this

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The deduced amino acid sequence of another ORF distal from bphE showed homology to transcriptional repressors of the GntR family (17), BphS of transposon Tn4371 (9) and ApsH of Comamonas testosteroni (18). This ORF had 74.4 and 37.0% identities to these repressors, respectively. The sequence also showed 40.2% identity to ORF0 from Pseudomonas pseudoalcaligenes KP707, which also belongs to the GntR family but exceptionally works as a positive regulator (19). Because the product of this ORF functioned as a regulator of the bph genes (see below), we designated it as bphS.

Repressive Function of the bphS Gene Product in KKS102—

The bphS gene of KKS102 was disrupted as indicated in Fig. 3A (for details see “Experimental Procedures”), and the effect of disruption on the expression of the bph genes was analyzed. The strains were grown in liquid culture with or without biphenyl, and their BphD activities were measured at 1, 3, 5, and 7 h after the addition of biphenyl. In the wild type KKS102, BphD activity was kept at a low level in the absence of biphenyl but not in its presence. In contrast, a high level of LacZ activity in the bphS disruptant (KLZ12ΔS) in the absence of biphenyl, and this level was even higher than that in the presence of biphenyl. These results clearly demonstrate that the bphS gene product negatively regulates bph gene expression in KKS102.

Characterization of a Promoter Located Upstream of bphE—

Because the bphE gene, the first gene in the bph gene cluster, is induced by biphenyl, we searched for a promoter in the upstream region of bphE. To identify the transcription start site in vivo, primer extension analysis was performed. When the primer BPHE10-29, which hybridized to the nucleotides 10–29 of the BphE-coding sequence, was used, a single band versus E. coli (GTGTTT) as the pE core promoter and the DNA region that includes the pE core promoter and the other elements involved in the transcriptional regulation as the pE promoter.

Identification of the DNA Region Required for Inducible Expression of the pE Promoter—We performed a deletion analysis of the bphE upstream region to define the sequence necessary for inducible promoter activity. A series of fusion constructs of the partially deleted 5’ region of the bphE and lacZ gene were integrated into the genome of KKS102 (see “Experimental Procedures” for details), and LacZ activity was measured in the presence or absence of biphenyl. By integrating the lacZ fusion constructs into the genome, the effect of difference in copy number of the reporter gene could be excluded from the measurement of the promoter activity. The results are summarized in Fig. 5A. First, pE is the sole promoter that resides within 1555 bp upstream of the bphE start codon. The same level of LacZ activity was observed in strain KLZ10 (containing up to 1555 bp from the bphE translation start site) and in strain KLZ12 (containing up to 387 bp from the bphE translation start site), indicating that there is no promoter activity be-
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The nucleotide sequence of the upstream region of the bph gene cluster. The deduced amino acid sequences of napBH and bphS are shown. Asterisks indicate the stop codons. The 4-bp terminal direct repeats and 16-bp terminal inverted repeats flanking napBH are indicated by boxes. The putative ribosome binding site of bphS is underlined. The predicted helix-turn-helix DNA binding motif in the BphS protein is shown with white letters under a black background. The ATG start codon of the divergently transcribed bphE gene is located at nucleotides 1–3.

Fig. 2. The nucleotide sequence of the upstream region of the bph gene cluster. The deduced amino acid sequences of napBH and bphS are shown. Asterisks indicate the stop codons. The 4-bp terminal direct repeats and 16-bp terminal inverted repeats flanking napBH are indicated by boxes. The putative ribosome binding site of bphS is underlined. The predicted helix-turn-helix DNA binding motif in the BphS protein is shown with white letters under a black background. The ATG start codon of the divergently transcribed bphE gene is located at nucleotides 1–3.

between nucleotides −388 and −1555. The deletion of the pE core promoter resulted in a markedly low level of LacZ activity (compare strains KLZ12 and KLZ9). Deletion of the sequence just upstream of the −35 box resulted in a decrease in LacZ activity (see strain KLZ3). This decrease may be due to deletion of the UP element, the third DNA element of the prokaryotic core promoter (20). Second, the DNA region ranging from −387 to −243 (where +1 is the bphE translation start site) is sufficient and necessary for promoter activity and the inducible expression because strain KLZ23 (which contains −387 to −243) showed enhanced LacZ activity that was much higher in the presence of biphenyl.

Specific Binding of BphS to the pE Promoter—The results described above suggested that the bphS gene product binds within the DNA region spanning from nucleotides −387 to −243. To determine whether the BphS binds to this DNA region, we conducted gel retardation experiments. The DNA region from −387 to −243 of the pE promoter was excised as an EcoRI-HindIII fragment from pYO12R and was end-labeled with 32P (Fig. 6A). The BphS protein was expressed in E. coli, and the CFE was used for the gel retardation assay. The retarded bands were observed only in the reactions containing BphS protein (Fig. 6B). There were two shifted bands at higher protein concentrations, suggesting that BphS binds to multiple sites within the fragment. When the nonradioactive DNA fragment was added to the binding reaction in excess of the 32P-labeled fragment, the retarded band was greatly reduced (Fig. 6B, lane 6). The addition of an unrelated DNA fragment did not affect the BphS binding (Fig. 6B, lane 7). The retarded band was not observed when CFE of E. coli harboring vector pHSG399 was used (Fig. 6B, lane 8). These results clearly indicate that BphS specifically binds to the DNA region spanning −387 to −243.

Inhibition of Binding of BphS to the pE Promoter by HOPDA—In our previous work, we demonstrated that HOPDA, the intermediate metabolite of the biphenyl degradation pathway, is the inducer molecule of bph genes in KKS102 (12). Here we performed a gel retardation assay in the presence of varying concentrations of HOPDA (0–0.5 mM). The amount of protein of CFE was fixed at 3 μg. Under this condition only one retarded band was observed (Fig. 6B, lane 3). As shown in Fig. 6C, the intensity of the retarded band was reduced in a concentration-dependent manner. In the presence of HOPDA at 0.5 mM, the retarded band disappeared almost completely (Fig. 6C, lane 6). In contrast, a saturated concentration of biphenyl (−0.1 mM) or 0.5 mM 2,3-dihydroxybiphenyl did not inhibit the binding. These results indicate that the BphS protein loses its ability to bind to the pE promoter in the presence of the inducer molecule HOPDA. This inhibition of binding of BphS to the pE promoter by HOPDA is consistent with the in vivo function of HOPDA as an inducer.

DNase I Footprinting Analysis—To obtain further information on the binding site of the BphS protein, DNase I footprinting analysis was carried out. A 183-bp pE promoter fragment containing the region from −387 to −243 was analyzed with both coding and noncoding strands (Fig. 7). The results of the DNase I footprinting are summarized in Fig. 7B. We identified four BphS binding sites and named them (beginning with the furthest upstream) BS I (binding site I), BS II, BS III, and BS IV. That a part of the DNA region, ranging from nucleotides −333 to −319 (BS I) and −315 to −299 (BS II) were protected from DNase I digestion (Fig. 7, lane 8). The addition of an unrelated DNA fragment did not affect the BphS binding (Fig. 7, lanes 7 and 9). When DNA labeled in the coding strand was incubated with a relatively low amount of CFE containing BphS, two regions ranging from nucleotides −333 to −319 (BS I) and −315 to −299 (BS II) were protected from DNase I digestion (Fig. 7A, lane 3). When DNA labeled in the noncoding strand was used under the same protein and DNA concentrations, DNA regions ranging from −329 to −315 (BS I) and −312 to −295 (BS II) were protected (Fig. 7A, lane 8). At higher protein concentrations, additional DNA regions from −296 to −281 (BS III) and −279 to −263 (BS IV) (Fig. 7A, lane 5, analyzing coding strand) and from −291 to −278 (BS III) and −274 to −260 (BS IV) (Fig. 7A, lane 10, analyzing noncoding strand) were protected. In Fig. 7, the DNA regions protected at low and high protein concentrations are indicated by thick and thin lines, respectively. No protection was observed when CFE of E. coli harboring vector plasmid was used (data not shown). These results demonstrate that the BphS protein binds to four sites near the pE core promoter and has greater affinity to the two upstream binding sites (BS I and BS II) than to the downstream sites (BS III and BS IV). That a part of the −10 box hexamer of the
pE promoter overlaps with BS I and was protected from DNase digestion suggests the essential role of BS I in BphS-mediated repression of the pE promoter.

**In Vivo Function of the BphS Binding Sites**—The results of the DNase footprinting demonstrated several interesting properties of the four binding sites. The protection of BS I and BS II, but not BS III and BS IV, under low protein concentration of BphS-containing CFE indicates that BS I and BS II have greater affinity for BphS protein than do BS III and BS IV.

To investigate the function of these two sets of BphS binding sites in the repression of the pE promoter in vivo, a series of promoter constructs was integrated into the genome of KKS102 as described under “Identification of the DNA Region Required for Inducible Expression of the pE Promoter” and assayed for promoter activity (Fig. 5B). The strain KLZ22, which had a construct with all four binding sites as well as the pE core promoter, showed low LacZ activity when grown in the absence of biphenyl. In contrast, the strain KLZ22, the integrated construct of which had BS I and BS II but lacked BS III and BS IV, showed high LacZ activity even in the absence of biphenyl. The LacZ activity in the absence of biphenyl was increased ~3-fold.
although it did not exceed that in the presence of biphenyl, by deletion of BS III and BS IV (compare strains KLZ23 and KL222 in the absence of biphenyl), demonstrating the in vivo function of BS III and BS IV. In strain KLZ21, where the integrated construct had no BphS binding site, LacZ activity in the absence of biphenyl was further elevated to the level observed in the strain KLZ15, whose integrated construct lacked BS I and BS II but had BS III and BS IV. This result indicates that the presence of BS I and BS II is essential for repression of the pE promoter.

In the strains KLZ12ΔS, KLZ21, and KLZ15, the presence of biphenyl resulted in lower lacZ activities than its absence. This might have been due to the cytotoxicity of biphenyl (21) and/or the catabolite-repressive effect on the pE promoter as a result of biphenyl assimilation. In conclusion, BS I and BS II were found to play an essential role in repression in vivo, and another set of two binding sites was shown to be functional, although its role was auxiliary.

Role of the pE Promoter in the Expression of Entire bph Genes—The results presented above demonstrate the role of BphS in the regulation of the pE promoter. Under “Repressive Function of the bphS Gene Product in KKS102,” we have demonstrated that disruption of the bphS gene resulted in constitutive expression of the bphA1, bphC, and bphE genes (Fig. 3). This suggests that BphS plays a central role in the regulation of entire bph genes and raises the following questions. Does the pE promoter drive the transcription of the entire bph gene cluster? Are there any other BphS-regulated promoters in the bph gene cluster? To address these questions, the pE promoter region from −242 to −400 in the strain KKS102 was replaced with a kanamycin resistance gene and a transcription terminator as depicted in Fig. 8. Since the absence of the pE promoter could hinder the accumulation of the inducer molecules and could lead to persistent repression of any promoters in the bph genes cluster by BphS, the effect of the deletion of the pE promoter was also tested in a strain with a bphS-disrupted background. The resulting KKS102 and KKSΔS derivatives were designated as KKSapE and KKSapEΔS, respectively. We then investigated the induction of BphD activity (Table I). The bphD gene is located relatively downstream in the bph genes cluster, and therefore BphD activity serves as an indicator of the presence of any intervening promoters. The BphD activities in KKSapE were very low irrespective of the presence or absence of biphenyl, even lower than that of the uninduced wild type strain. In addition, the bph mRNA level detected by the bphA1, bphA4, bphC, or bphD-specific probe was low, and no induction by biphenyl was observed. We thus concluded that pE is the primary promoter for the transcription of most bph genes.

DISCUSSION

The bphS Gene Product Is a Negative Regulator of bph Genes—In the bphS gene disrupted, expression of BphD and LacZ reporter activity and amounts of bph gene transcripts were elevated even in the absence of biphenyl to the level of those in the induced wild type strain, indicating that the bphS gene product plays an essential role in repression of the bph genes. This repression results from the direct action of the bphS gene product at the pE promoter because the BphS protein specifically bound to the pE promoter and deletion of BphS binding sites led to constitutive production of LacZ activity. The repressor function of BphS is consistent with the fact that BphS belongs to the GntR family of transcriptional repressors.

pE Promoter, an Essential Promoter for Transcription of bph Genes—The LacZ reporter assay of the upstream region of bphE revealed that only one promoter, which was designated the pE promoter, exists in the region. Elimination of the pE promoter resulted in weak and constitutive production of BphD activity as well as of bphA1, bphA4, bphC, and bphD transcripts, demonstrating that the pE promoter plays an essential role in the expression of bph genes and that bph genes (at least

![Deletion analysis of the bphE upstream region. A, LacZ activity in a series of strains harboring the fusion of the 5' region of bphE and the lacZ gene in the chromosome. The DNA region fused with lacZ is shown with white bars at the left. The numbers in the figure represent the marginal nucleotide positions of the fusion constructs (1 + represents the bphE translation start codon). Each strain was incubated in 1/3 LB, and LacZ activity was measured in the same conditions as described in Fig. 3D. LacZ activity in the cells incubated with (filled bars) or without (empty bars) biphenyl is shown on the right. B, nucleotide sequence around the pE promoter. Putative BphS recognition sequences deduced from DNase I footprinting are boxed. The four binding sites are also denoted in the figure by black boxes.](http://www.jbc.org/content/361/15/36151/F5.large.jpg)
Protein concentrations were (in lane 8) 0.1 (lanes 5 and 6), 0.3 (lanes 3, 4, and 7), and 1.0 (lane 4). A 40-fold excess of the unlabeled fragment (lane 6) or 1 ng of unrelated fragment (sermon sperm DNA) (lane 7) was added to the mixture. Unbound free DNA is marked F; bound DNA is marked C1 or C2. C, binding of BphS in the absence of the inducer molecule. 1 ng of labeled fragment was incubated with 0.3 μg of CFE of E. coli harboring pH701 (a plasmid coding bphS) (lanes 2–7), or CFE of E. coli harboring pHSG399 (vector for pH701) (lane 8). Protein concentrations were (in μg/10 μl of reaction mixture) 0.05 (lane 2), 0.3 (lanes 3, 6, and 7), and 1.0 (lane 4), 5.0 (lanes 5 and 8). A 40-fold excess of the unlabeled fragment (lane 6) or 1 ng of unrelated fragment (sermon sperm DNA) (lane 7) was added to the mixture. Unbound free DNA is marked F; bound DNA is marked C1 or C2. C, binding of BphS in the presence of the inducer molecule. 1 ng of labeled fragment was incubated with 0.3 μg of CFE of E. coli harboring pH701. Lanes 1–6 contain the following concentration of HPDNA: 0, 5, 25, 50, 250, and 500 μM. Lane 7 contains the saturated concentration of biphenyl (0.1 mM). Lane 8 contains 2,3-dihydoxybiphenyl (500 μM).

from bphE to bphA4) constitute an operon. Although some other parts of the nucleotide sequence may exert promoter activity in the long (12-kb) bph gene cluster, it seems that such promoter activities are trivial compared with the promoter activity of the pE promoter. For example, we detected promoter activity upstream of the bphA1 gene, but fusion with the lacZ genes showed that the activity was significantly lower (20 times lower) than that observed for the pE promoter (data not shown). In conclusion, the pE promoter is the primary promoter driving transcription of the bph operon.

BphS Binding to the pE Promoter—In vivo and in vitro experiments showed specific binding of BphS protein to the pE promoter, indicating that the BphS protein plays an essential role in regulation of the bph operon because the pE promoter is the primary promoter involved in expression of the bph operon.

To identify additional binding sites for the BphS protein that might be involved in repression, we performed a gel retardation assay. We used various DNA fragments derived from the bphE upstream region as well as the bphE-coding region (from −2262 to −396 where +1 represents the bphE translation start codon), but we did not identify any additional BphS binding sites (data not shown).

Consensus Operator Sequence of BphS Protein—DNase footprint analysis identified four binding sites for BphS just downstream of the pE promoter. The binding sites were named, beginning with the most upstream site, BS I, BS II, BS III, and BS IV (Fig. 7B). The gel retardation assay revealed that the affinity of BphS to these sites is greater in the order of BS II > BS I > BS IV > BS III.2 We also tested an inverted repeat sequence found in the vicinity of the promoter of the bph genes on Tn4371 (9) (Fig. 9) and found that BphS of KKS102 bound to this sequence at affinity greater than to BS II.2 The operator sequences for GntR family members have been suggested to contain perfect or imperfect inverted repeat sequences (22). BS I contains an imperfect inverted repeat sequence and BS II contains a perfect inverted repeat sequence, while BS III and BS IV have no distinct inverted repeat sequence. Alignment of the three stronger binding sites with distinct inverted repeat sequences (BS I, BS II, and the inverted repeat sequence found in Tn4371) identified the consensus sequence of AN12T (Fig. 9) in an AT-rich symmetric sequence, which was reminiscent of the binding motif (TN11A) for LysR-type transcriptional regulators (23). Conservation of A and T nucleotides separated by 12 base pairs seems important based on the fact that, in several DNA binding proteins that use a helix-turn-helix motif for binding, two recognition helices in the dimers are separated about one turn of DNA helix long (24). In the DNA sequences of BS III and BS IV, the AN12T motifs were found, although these binding sites were less symmetric, which might be reflected in the comparably weak binding affinity for BphS (Fig. 7B). Further investigations will be needed to clarify the importance of the AN12T motif of BphS binding sites.

The AT content around the binding site was 78.4% in the 74 nucleotides from −333 to −260), and this value was very high for the bph genes in which the average GC content was 62%. The abundance of AT base pairs may have helped to form a structure favored by BphS binding.

Repression Mechanism Mediated by Four BphS Binding Sites and BphS Protein—The data obtained from the promoter-lacZ fusion indicates that the proximal two binding sites (BS I and BS II), as well as the distal sites (BS III and BS IV), are functional in vivo for repression and that BS I and BS II play a primary role in repression. How, then, do these four binding sites and the BphS protein mediate transcriptional repression? Protection from DNase I cleavage of part of the −10 box by BphS indicates that the BphS protein bound at BS I masks the −10 box of the pE promoter and prevents the access of RNA polymerase as has been described in many other cases, as for example, the binding of the phage λ cl repressor to the operators O1 and O2 (25) and also the binding of LacI to the O1 operator of the lac promoter (26). The role of BphS proteins bound at BS II may be to enhance repression through stabilization of BphS binding to BS I through protein-protein interaction. BphS bound to BS I may also stabilize binding of BphS to BS II, and thus the binding of BphS repressors to these sites could be cooperative. In support of this possibility, only two species of retarded band were observed in the gel retardation assay; one might represent binding to BS I and BS II and the other might represent additional binding to BS III and BS IV, suggesting preferable binding of BphS protein to each set of operator sites. In addition, the finding of simultaneous protection of BS I and BS II and of BS III and BS IV in the DNase footprinting analysis was consistent with the notion that the BphS protein has a cooperative binding property. In our recent study, purified His6-tagged BphS protein was shown to bind to a DNA fragment containing both BS I and BS II with 10 times

2 Y. Ohtauba, M. Delawary, K. Kimbara, M. Takagi, A. Ohta, and Y. Nagata, unpublished data.
more efficiency than to a DNA fragment containing only BS II (as mentioned under “Consensus Operator Sequence of BphS Protein,” the affinity for the BphS protein is stronger than that of BS I), supporting the cooperative binding of BphS to BS I and BS II.2 In a recent review on bacterial transcriptional regulation, possible cooperative interaction of GntR dimer pairs was suggested because the candidate binding sites for GntR occurred in pairs in several cases (27). A GntR family protein, AphS from C. testosteroni TA441, binds to two sites in the promoter region, although the implications of the presence of these two binding sites are not known (18).

It has been reported that multiple binding sites for other types of negative regulators are necessary for efficient repression. For example two GalR dimers bound to two operators have been shown to interact with each other and repress transcription of the gal operon (28). And LacI binds to three operators and cooperates in repression (29).

Derepression of BphS-mediated Repression by HOPDA—We demonstrated in vitro that BphS protein binds specifically to the pE promoter and that the binding affinity decreases in the presence of HOPDA. This feature of the BphS protein enables high levels of the expression of bph genes only when the intermediate of the biphenyl degradation pathway is present. This finding is well consistent with the previous finding that

![Figure 7](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**FIG. 7.** DNase I footprinting of BphS binding to the pE promoter region. A, DNase I footprinting of the DNA fragment labeled for the coding (lanes 1–5) and noncoding (lanes 6–10) strands. Lanes 1 and 6, Maxam-Gilbert G and A reaction products. Lanes 2 and 6, DNase I digests in the absence of CFE. Lanes 3, 4, and 5 and lanes 8, 9, and 10, DNase I digests of the reaction mixture containing the following amounts of protein in CFE of E. coli harboring plasmid pH701: 1 μg (lanes 3 and 8), 5 μg (lanes 4 and 9), and 20 μg (lanes 5 and 10). The thick lines indicate the regions protected from DNase I digestion when a lower amount of CFE (1 μg) was used. The thin lines indicate the regions protected when a higher amount of CFE (5 or 20 μg) was used. Numbers at the left indicate nucleotide positions relative to the translation start site of bphE. B, summary of footprinting data of BphS binding to the pE promoter. The protected regions are indicated by thick and thin lines as described above.

**TABLE I**

| Strains | Addition of biphenyl | BphD activity units/mg protein |
|---------|----------------------|-----------------------------|
| KKS102  | +                    | 68.8                        |
| -       |                      | 18.1                        |
| ΔpE     | +                    | 7.7                         |
| -       |                      | 10.4                        |
| ΔpEdΔphS| +                    | 7.6                         |
| -       |                      | 8.4                         |
| ΔphS    | +                    | 75.5                        |
| -       |                      | 88.3                        |
Regulation of bph Genes in Pseudomonas sp. KKS102

The increase in bph gene products results in further elevation of the Biodegradation of HOPDA, the inducer of bph genes in vivo. The question of the mechanisms by which gene expression occurs only under particular circumstances is of great interest. A model depicting the molecular aspect of bph gene regulation is as follows. In KKS102 cells, the BphS protein binds to its binding sites and inhibits transcription from the pE promoter. In this repressed state, binding of BphS to BS I plays a central role, and BphS protein bound to the other sites helps to stabilize the BphS protein bound at BS I. When the cells encounter biphenyl, biphenyl is converted to HOPDA by bph gene products that are somehow maintained at the basal level, leading to dissociation of the BphS protein from the operator DNA and to subsequent active transcription initiation at the pE promoter. The increase in bph gene products results in further elevation of the HOPDA concentration. In conclusion, the BphS protein is a key component of the molecular switch regulating expression of the bph operon in KKS102. It regulates the pE promoter that is essential for expression of the bph operon.

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FIG. 9. Comparison of binding sites. Binding sites I, II, III, and IV as well as the BphS binding sequence from Tn4371 are shown. Conserved A and T bases are shown by white letters on a black background. Bases that are involved in symmetry are in bold type. The vertical line indicates the axis of symmetry.
BphS, a Key Transcriptional Regulator of bph Genes Involved in Polychlorinated Biphenyl/Biphenyl Degradation in Pseudomonas sp. KKS102
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