

Characterization of Transcriptional Regulatory Genes for Biphenyl Degradation in *Rhodococcus* sp. Strain RHA1

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Transcription of the *bphA1A2A3A4C1B* genes, which are responsible for the conversion of biphenyl and polychlorinated biphenyl to the *meta*-cleavage products in *Rhodococcus* sp. strain RHA1, was examined. The *bphA1* promoter (P_{bphA1}) was identified and was shown to promote transcription induction by biphenyl and ethylbenzene. An 8.8-kb HindIII fragment that promotes transcription induction of P_{bphA1} in *Rhodococcus erythropolis* IAM1399 was isolated from the region downstream of *bphB* by using a reporter plasmid containing P_{bphA1} . Analysis of the nucleotide sequence of this fragment revealed a set of putative two-component regulatory system genes, which were designated *bphS* and *bphT*. Deletion analysis of the 8.8-kb HindIII fragment indicated that *bphT* is responsible for the basal activation of P_{bphA1} and that both *bphS* and *bphT* are required for the elevated basal activation of and transcriptional induction by biphenyl of P_{bphA1} . These results support the notion that *bphS* and *bphT* encode a sensor kinase and a response regulator, respectively, of a two-component regulatory system. The *bphS* and *bphT* genes promote transcriptional induction by a variety of aromatic compounds, including biphenyl, benzene, alkylbenzenes, and chlorinated benzenes. A promoter activity assay and reverse transcription (RT)-PCR analysis revealed a weak constitutive promoter in the adjacent region upstream of *bphS*. RT-PCR analysis indicated that there is induced transcription of *bphA1* through *bphT*, in which P_{bphA1} is thought to take part. An insertionally inactivated *bphS* mutant, SDR1, did not grow on biphenyl. Growth was restored by introduction of an intact *bphS* gene into SDR1. These results indicate that at least *bphS* is indispensably responsible for the growth of RHA1 on biphenyl.

Polychlorinated biphenyls (PCBs) are man-made compounds that have been widely used for industrial purposes due to their exceptional stability. As a consequence, PCBs have caused widespread contamination in the environment. With the goal of remediating contaminated environments, microorganisms that can degrade PCBs have been isolated, and their degradative genes have been characterized (2, 6, 13, 21). These microorganisms cometabolize PCBs through the biphenyl metabolic pathway.

In the aerobic biphenyl metabolic pathway, biphenyl is transformed to benzoate and 2-hydroxypenta-2,4-dienoate by sequential actions of a multicomponent biphenyl dioxygenase (BphA encoded by *bphA1A2A3A4*) (Fig. 1), a dihydrodiol dehydrogenase (BphB encoded by *bphB*), a 2,3-dihydroxybiphenyl dioxygenase (BphC encoded by *bphC*), and a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD encoded by *bphD*). 2-Hydroxypenta-2,4-dienoate is further metabolized to pyruvate and acetyl coenzyme A by 2-hydroxypenta-2,4-dienoate hydratase (BphE encoded by *bphE*), 4-hydroxy-2-oxovalerate aldolase (BphF encoded by *bphF*), and acetaldehyde dehydrogenase (BphG encoded by *bphG*). These *bph* gene-encoded enzymes are usually induced by biphenyl and are involved in the cometabolism of PCBs.

We isolated a gram-positive biphenyl and PCB degrader, *Rhodococcus* sp. strain RHA1, which has a great capacity to degrade highly chlorinated PCBs (31). In contrast to gram-negative PCB degraders, which have a single set of biphenyl-

PCB degradation genes clustered in a single locus (11, 12), RHA1 possesses diverse biphenyl-PCB degradation genes that encode multiple isozymes for each metabolic step and are distributed among multiple clusters (14, 28, 38). Most of these genes are located on linear plasmids, designated pRHL1 and pRHL2 (32). The *bphA1A2A3A4C1B* genes involved in the upper degradation pathway are located on pRHL1, and all of the other *bph* genes except *bphGF1E1* and *bphC5* are located on pRHL2. *bphGF1E1* and *bphC5* are on the chromosome. *bphA1* and *bphC1* were found to be transcriptionally induced by biphenyl (14, 28). Gene disruption and enzyme activity analyses suggested that at least the *bphA1* and *bphC1* genes play major roles in biphenyl-PCB metabolism (10, 20).

Recently, several transcriptional regulatory systems for biphenyl and PCB degradation pathway genes have been described. In *Ralstonia eutropha* A5, it has been suggested that the *bph* gene cluster, *bphEFGA1A2A3BCD*, forms an operon transcribed from a σ^{70} promoter, which is negatively regulated by a *bphS* gene-encoded repressor (23). Transcription of the *bph* gene cluster *bphEGF(orf4)A1A2A3BCD(orf1)A4* in *Pseudomonas* sp. strain KKS102 has been found to be regulated by BphS (24). In *Pseudomonas pseudoalcaligenes* KF707, it has been found that the ORF0 protein is involved in the regulation of a lower-pathway *bph* gene operon containing *bphX0X1X2X3D* (37). In *Burkholderia* sp. strain LB400, the ORF0 protein was found to be involved in the regulation of transcription from a promoter upstream of *bphA1* (3). All these regulators of the biphenyl-PCB degradation pathway in gram-negative bacteria belong to the GntR family of transcriptional regulators. On the other hand, it has been suggested that in gram-positive bacteria, a two-component regulatory system encoded by *bpdST* is involved in biphenyl-PCB metabolism

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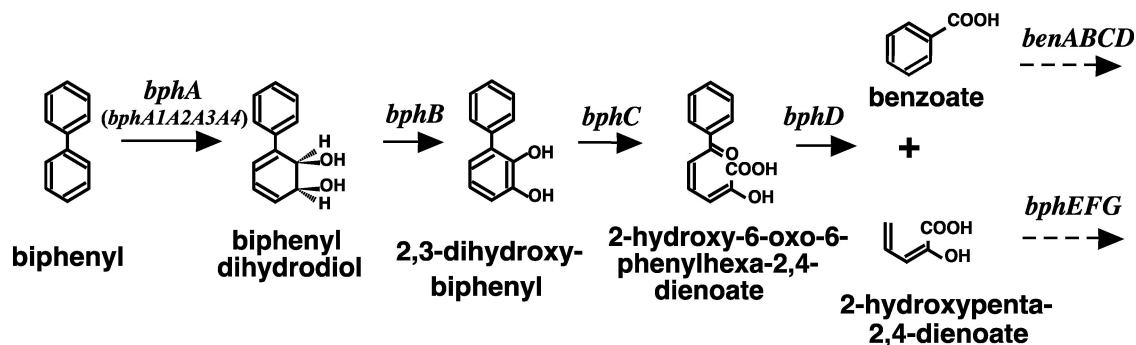


FIG. 1. Proposed pathway for aerobic bacterial degradation of biphenyl in *Rhodococcus* sp. strain RHA1. The gene(s) responsible for each enzyme step is indicated above an arrow and is described in the text.

only in *Rhodococcus* sp. strain M5 (15). The biphenyl degradation genes of RHA1 are not as similar to those of M5, and the gene organization is distinct from that of M5. In addition, the genes are distributed on linear plasmids. Thus, the regulatory system for biphenyl-PCB metabolism in gram-positive bacteria has attracted much interest.

In the present study we focused on regulation of the *bphA1A2A3A4CIB* genes, which are known to be expressed and to be responsible for biphenyl and PCB degradation in the gram-positive PCB degrader strain RHA1. Here we describe cloning of the two-component regulatory system for *bph* gene transcription in RHA1 and present evidence that this system is involved in biphenyl metabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Rhodococcus* sp. strain RHA1 was grown in Luria-Bertani (LB) medium (10 g of Bacto Tryptone [Difco] per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter) and W minimal medium (20) containing one of the following carbon sources: 0.2% biphenyl, 0.2% sodium benzoate, 0.2% sodium succinate, ethylbenzene, toluene, benzene, or *ortho*-xylene. Ethylbenzene, toluene, benzene, and *ortho*-xylene were supplied in the vapor phase. The host strains, *Rhodococcus erythropolis* IAM1399 (= ATCC 15963) and *Escherichia coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'[traD36 proAB⁺ lacI^q lacZΔM15]*) were grown in LB medium. The plasmids used in this study are listed in Table 1.

DNA manipulations and analysis. All the DNA techniques used, including gene cloning, nucleotide sequencing, electrotransformation (electroporation), and computer analysis of DNA sequences, have been described previously (19, 20, 38).

RNA slot blot analysis. RHA1 total RNA was prepared as described previously (38). Two micrograms of total RNA was blotted onto a nylon membrane (Hybond N; Amersham International plc, Buckinghamshire, United Kingdom) by using a slot blot apparatus (Bio-Rad, Richmond, Calif.). Probes were labeled as described in the digoxigenin system manual (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The conditions used for prehybridization, hybridization, washes, and detection were the conditions recommended in the instructions supplied by Boehringer Mannheim for the digoxigenin kit.

Primer extension. The 5' end of *bphA1* mRNA was mapped by using oligonucleotide primer PEXA1 (5'-TACGAGTTCAGCGATGTC CG-3', corresponding to nucleotides 215 to 235 relative to the identified transcriptional start site, P1). The primer was end labeled by using T4 polynucleotide kinase (Nippon Gene Co., Tokyo, Japan) with [γ -³²P]ATP (Amersham). Two picomoles of the primer was incubated with 20 to 50 μ g of RNA in 10 μ l of hybridization buffer (10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 0.15 M KCl) at 65°C for 90 min and was allowed to cool. After addition of 30 μ l of concentrated reverse transcriptase buffer (containing each deoxynucleoside triphosphate at a concentration of 0.33 mM, 20 mM Tris-HCl [pH 8.3], 10 mM MgCl₂, 100 μ g of actinomycin D per ml, and 5 mM dithiothreitol) and 20 U of avian myeloblastosis virus reverse transcriptase (Takara Shuzo Co., Ltd., Kyoto, Japan), the mixture was incubated at 42°C for 60 min. The DNA was recovered by ethanol precipitation, after which

it was dissolved in 5 μ l of formamide loading buffer, and an aliquot was analyzed in an 8% sequencing gel.

RT-PCR. Reverse transcription (RT)-PCR was performed by using a BcaBEST RNA PCR kit (Takara) for the usual conditions or a ReverTra Dash kit (TOYOBO Co., Ltd., Osaka, Japan) for intensive conditions, as described in the manufacturer's protocol. RT-PCR with ReverTra Dash generates more PCR product and provides superior sensitivity. One to two micrograms of total RNA was reverse transcribed with random primers and PCR amplified with each primer set for 30 cycles at an annealing temperature of 55°C. RNA samples were concurrently analyzed in PCR mixtures without reverse transcriptase to verify the absence of contaminating genomic DNA. PCR mixtures were analyzed on a 2% agarose gel. The primers used for each intergenic region are listed in Table 2.

Construction of a promoter probe vector. A promoter probe vector was constructed by using a *Rhodococcus-E. coli* shuttle vector, pK4 (9), and *luxAB* luciferase structural genes from *Vibrio harveyi*, and it was designated pKLA1 (see Fig. 3A). pKLA1 has a unique *Sa*I site for cloning of a DNA fragment containing a promoter region. The promoter fragments were blunt ended and inserted into the blunt-ended *Sa*I site of pKLA1.

Luciferase assay. Recombinant plasmids of pKLA1 were introduced into RHA1 or IAM1399 cells by electroporation. Transformant cells grown on LB medium containing kanamycin (50 μ g/ml) were washed with 50 mM sodium phosphate buffer (pH 7.0) and suspended in 10 ml of 0.2 \times LB medium (2 g of Bacto Tryptone per liter, 1 g of yeast extract per liter, 5 g of NaCl per liter) containing kanamycin (50 μ g/ml) at an *A*₆₀₀ of 1.0. Each cell suspension was incubated at 30°C for 5 h in the absence or presence of an inducer compound. Solid compounds were each supplied as a powder at a final concentration of 0.2%, and volatile compounds were supplied in vapor. The cell suspension was then diluted 1:10 in lux buffer (25), and a 100- μ l aliquot was mixed with 390 μ l of lux buffer. After addition of 10 μ l of 0.1% (vol/vol) 1-decanol in lux buffer to the resulting 490 μ l of diluted cell suspension, the luciferase activity was measured with a luminometer (Lumitester K-100; Kikkoman, Noda, Japan). The total light generated during the initial 15 s was recorded, and the activity was expressed in light units per milliliter of culture per unit of *A*₆₀₀.

Detection of gene products. An *Apa*I-BglIII fragment of pG1013F carrying *bphT* was inserted between *Apa*I and *Bam*HI sites of pBSL, yielding plasmid pBAB14. Then the 4.8-kb *Apa*I fragment of pG1013F carrying the 5' part of *bphS* was cloned into the *Apa*I site of pBAB14. The resulting plasmid, pBAB62, contained the whole *bphST* region. The *Spe*I fragments of pBAB14 and pBAB62 carrying *bphT* and *bphST*, respectively, were blunt ended and cloned into the blunt-ended *Bam*HI site of pET21(+). The resulting plasmids, pETT14 and pETST62, respectively, were introduced into *E. coli* BL21. Transformants were grown in LB medium containing ampicillin (50 μ g/ml) at 37°C for 2 h and then for 5 h in the presence of isopropyl- β -D-thiogalactopyranoside at a final concentration of 1 mM. The cells were washed with 0.5 ml of sodium phosphate buffer (pH 7.0) and resuspended in 0.1 ml of lysis buffer consisting of 100 mM Tris-HCl (pH 6.8), 10% (vol/vol) glycerol, 5% sodium dodecyl sulfate (SDS), and 1 mM 2-mercaptoethanol. Aliquots were boiled for 10 min prior to electrophoresis on an SDS-7% polyacrylamide gel. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (19).

In vitro expression. In vitro expression of a cloned gene was carried out with the *E. coli* S30 extract system (Promega, Madison, Wis.) used according to the manufacturer's protocol. The polypeptides were labeled with [³⁵S]methionine

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or origin
Strains		
<i>Rhodococcus</i> sp. strain RHA1	PCB degrader, BPH ⁺	30
<i>Rhodococcus</i> sp. strain SDR1	<i>bphS</i> mutant of strain RHA1, BPH ⁻	This study
<i>R. erythropolis</i> IAM1399 (= ATCC 15963)	Wild type, BPH ⁻	IAM culture collection ^b
Plasmids		
pBluescript II KS+	Cloning vector, Ap ^r	Stratagene
pG1013F	pBluescript II KS+ with 8.8-kb HindIII fragment of RHA1 carrying <i>bphST</i>	This study
pBSL	pBluescript II KS+ with new SpeI site in place of KpnI site	This study
pBAB14	pBSL with 1.4-kb ApaI-BglII fragment of RHA1 carrying <i>bphT</i>	This study
pBAB62	4.8-kb ApaI fragment carrying <i>bphS</i> inserted into pBAB14	This study
pBAB62ΔSacI	1.0-kb SacI fragment carrying <i>bphT</i> deleted from pBAB62, leaving <i>bphS</i>	This study
pBAS52	SacI site of pBAB62ΔSacI converted to a SpeI site	This study
pET21(+)	Expression vector, Ap ^r	Novagen
pETT14	pET21(+) with SpeI fragment of pBAB14 carrying <i>bphT</i>	This study
pETST62	pET21(+) with SpeI fragment of pBAB62 carrying <i>bphST</i>	This study
pKLA1	Promoter probe vector containing luciferase structural gene, <i>luxAB</i> from <i>V. harveyi</i> , Km ^r (<i>neo</i>)	38
pKLAF1	pKLA1 with 1.4-kb XhoI-BamHI fragment of RHA1 carrying promoter region of <i>bphA1</i>	This study
pKA851	pKLAF1 with 8.8-kb HindIII fragment of RHA1 carrying <i>bphST</i>	This study
pKAD1-12	pKLAF1 with deletion derivatives of 8.8-kb HindIII fragment of RHA1	This study
pKLAS1	pKLAF1 with 5.4-kb SacI fragment of RHA1 carrying <i>bphS</i>	This study
pKLAST1	pKLAF1 with 6.2-kb ApaI-BglII fragment of RHA1 carrying <i>bphST</i>	This study
pKLASF	pKLA1 with 0.9-kb PstI-ClaI fragment of RHA1 carrying promoter region of <i>bphS</i> ; direction of <i>bphS</i> is identical to that of <i>luxAB</i> reporter gene	This study
pKLASR	pKLA1 with 0.9-kb PstI-ClaI fragment of RHA1 carrying promoter region of <i>bphS</i> ; direction of <i>bphS</i> is opposite that of <i>luxAB</i> reporter gene	This study
pUC-KmD	Gene disruption plasmid, Km ^r (<i>aphII</i>)	29
pUKSD	<i>bphS</i> disruption plasmid pUC-KmD with 1.3-kb ClaI fragment carrying <i>bphS</i> internal fragment of RHA1	This study
pFAJ2574	<i>Rhodococcus-E. coli</i> shuttle vector, Cm ^r	5
pFJS1	pFAJ2574 with 5.2-kb SpeI fragment of pBAS52 carrying <i>bphS</i>	This study

^a BPH⁺, growth on biphenyl; BPH⁻, no growth on biphenyl.

^b IAM, Institute of Applied Microbiology.

(Amersham). The labeled proteins were separated and detected by SDS-7% PAGE and autoradiography, respectively.

Deletion analysis. Restriction fragments of pG1013F (see Fig. 6), which were extracted and blunt ended by using T4 DNA polymerase, were cloned into the blunt-ended KpnI site of pKLAF1. Transcription of the *bphST* genes was started from the kanamycin resistance gene promoter of the pKLAF1 vector. The blunt-

ended SpeI fragment of pBAB62 containing *bphST* was inserted into the blunt-ended KpnI site of pKLAF1, yielding plasmid pKLAST1. To construct the frameshift derivatives pKAD11 and pKAD12, pG1013 was linearized with XmaI and Sse8387I, respectively, and religated after filling in of the ends. Each HindIII fragment containing the *bphST* genes of pG1013 derivatives was blunt ended and inserted into the blunt-ended KpnI site of pKLAF1. To obtain the *bphS* plasmid pFJS1, pBAB62ΔSacI was constructed by deleting the SacI fragment from pBAB62, which generated an ApaI-ApaI-SacI insert containing *bphS* in pBAB62ΔSacI. An SpeI linker was inserted into the blunt-ended SacI site of pBAB62ΔSacI to obtain pBAS52. pFJS1 was constructed by inserting an SpeI fragment containing the *bphS* insert of pBAS52 into the XbaI site of pFAJ2574, which is a chloramphenicol-resistant *E. coli-Rhodococcus* shuttle vector.

Gene disruption and complementation. To disrupt the *bphS* gene, a 1.3-kb ClaI fragment containing the internal region of *bphS* was blunt ended with T4 DNA polymerase and inserted into the SmaI site of pUC-KmD (29), which is a kanamycin-resistant vector and cannot replicate in *Rhodococcus* strains. The resulting plasmid, pUKSD, was introduced into RHA1 cells by electroporation. A single crossover between *bphS* and pUKSD sequences was expected to generate tandemly duplicated *bphS* sequences, leaving a vector containing a kanamycin resistance gene (*aphII*) between them. Transformants were selected on LB agar plates containing kanamycin (50 μg/ml) and were subjected to a Southern hybridization analysis in order to examine insertion of pUKSD into the *bphS* gene in RHA1 by single crossover. The insertion mutant obtained was designated SDR1.

To perform *bphS* gene complementation in SDR1, pFJS1 was introduced into SDR1 by electroporation. A transformant was isolated on an LB agar plate containing chloramphenicol (20 μg/ml). SDR1/pFJS1 and SDR1/pFAJ2574 cells grown in LB medium were washed and resuspended in W minimal medium

TABLE 2. Primers used for RT-PCR

Primer	Sequence	Region amplified
1-F	5'-TGGATCTATCGATGACGTTCC-3'	<i>bphS</i> internal
1-R	5'-GAGGAGAACGAGCTGATTGG-3'	<i>bphS</i> internal
2-F	5'-CGGCGGAAACGACCTGTAAG-3'	<i>bphB-bphS</i>
2-R	5'-AGCACCTCACGGTGTGCGATTTC-3'	<i>bphB-bphS</i>
3-F	5'-TCGGACAATCCCGATTACC-3'	<i>bphA1-bphA2</i>
3-R	5'-AGAAGTCTCGATCTCGTGC-3'	<i>bphA1-bphA2</i>
4-F	5'-GACCAGAGCACAATTCTCTCC-3'	<i>bphA2-bphA4</i>
4-R	5'-ATAGCTTCCGAACGCAG-3'	<i>bphA2-bphA4</i>
5-F	5'-GAGTTGCGCGAAGTGAATCG-3'	<i>bphA4-bphC1</i>
5-R	5'-AGATCCGCAACCTCGAAGC-3'	<i>bphA4-bphC1</i>
6-F	5'-GTGCGCTACGACAAGATCAGC-3'	<i>bphC1-bphB</i>
6-R	5'-GCCGAAGTCTGGTGGCAAGC-3'	<i>bphC1-bphB</i>
7-F	5'-AGACACCCTGCGGGCCGTGG-3'	<i>bphS-bphT</i>
7-R	5'-CGAGGCCGTTGACGTCGGACAGT-3'	<i>bphS-bphT</i>
8-F	5'-GAGGTGCCGATCAGACGATG-3'	<i>bphT</i> internal
8-R	5'-CGGAGGCGATCAGCTTCATT-3'	<i>bphT</i> internal

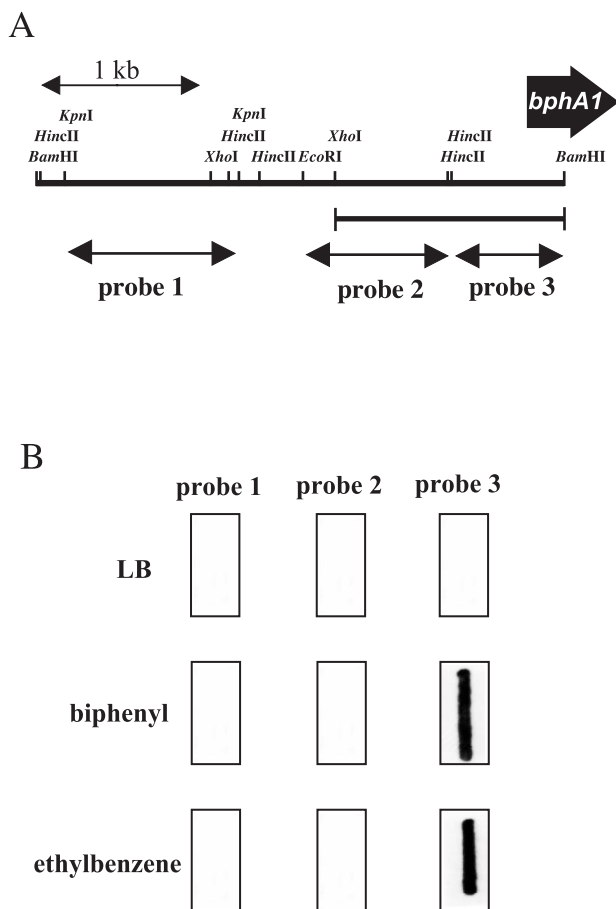


FIG. 2. Characterization of the *bphA1* promoter. (A) DNA probes used for RNA slot blot hybridization. A 1.1-kb KpnI fragment (probe 1), a 0.9-kb EcoRI-HincII fragment (probe 2), and a 0.7-kb HincII-BamHI fragment (probe 3) of the *bphA1* upstream region were used to localize the promoter region of *bphA1*. The position of the 1.4-kb XhoI-BamHI fragment containing the *bphA1* promoter is indicated by a line below the physical map. (B) RNA slot blot hybridization analysis of *bphA1* transcripts in *Rhodococcus* sp. strain RHA1. Two micrograms of total RNA from RHA1 cells grown in LB medium or on a substrate as a sole source of carbon in W minimal medium was blotted onto a nylon membrane and hybridized with digoxigenin-labeled probes 1, 2, and 3.

containing 0.2% biphenyl. The optical density at 600 nm was adjusted to 0.2, and the cell suspension was incubated at 30°C with shaking to examine the growth on biphenyl.

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB107790.

RESULTS

Expression of the *bphA1* promoter in RHA1. To locate the promoter region of the *bphA1* gene in RHA1, transcription of the region upstream of the *bphA1* gene was examined by RNA slot blot hybridization analysis by using three separate probes for the *bphA1* upstream region (Fig. 2A). Total RNA was extracted from the RHA1 cells grown on LB medium, biphenyl, or ethylbenzene and blotted on a membrane. Neither of the distal upstream probes (probes 1 and 2) hybridized to the total RNA from the cells grown on LB medium, biphenyl, or

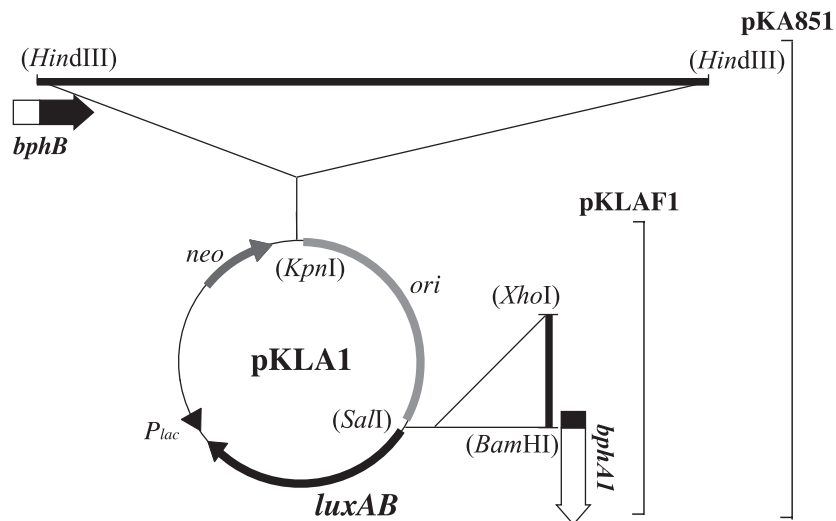
ethylbenzene (Fig. 2B). The proximal upstream probe, probe 3, hybridized to the RNAs from both biphenyl- and ethylbenzene-grown RHA1 cells. It did not hybridize to RNA from the cells grown on LB medium. These results suggest that the biphenyl- and ethylbenzene-inducible *bphA1* promoter (P_{bphA1}) is located in the 1.4-kb XhoI-BamHI fragment of the *bphA1* upstream region, which includes probe 3 and its adjacent upstream region.

To instantly examine the expression of P_{bphA1} , the 1.4-kb XhoI-BamHI fragment was inserted into the SalI site of a promoter probe vector, pKLA1 (38) (Fig. 3A). The SalI site in pKLA1 precedes a reporter gene that encodes a LuxAB luciferase of *V. harveyi*. The resulting plasmid was designated pKLA1 (Fig. 3A). RHA1 cells harboring pKLA1 were grown on 0.2× LB medium in the absence or presence of biphenyl, ethylbenzene, toluene, benzene, *ortho*-xylene, succinate, or benzoate and were subjected to the luciferase assay (see Materials and Methods). Transcription from P_{bphA1} was activated by biphenyl, ethylbenzene, toluene, benzene, and *ortho*-xylene (Fig. 3B). Transcription from P_{bphA1} was induced by neither succinate nor benzoate. The luciferase activity conferred by pKLA1 in the cells grown in 0.2× LB medium was, however, 85-fold higher than the luciferase activity of pKLA1, suggesting that P_{bphA1} has basal promoter activity in addition to inducible activity.

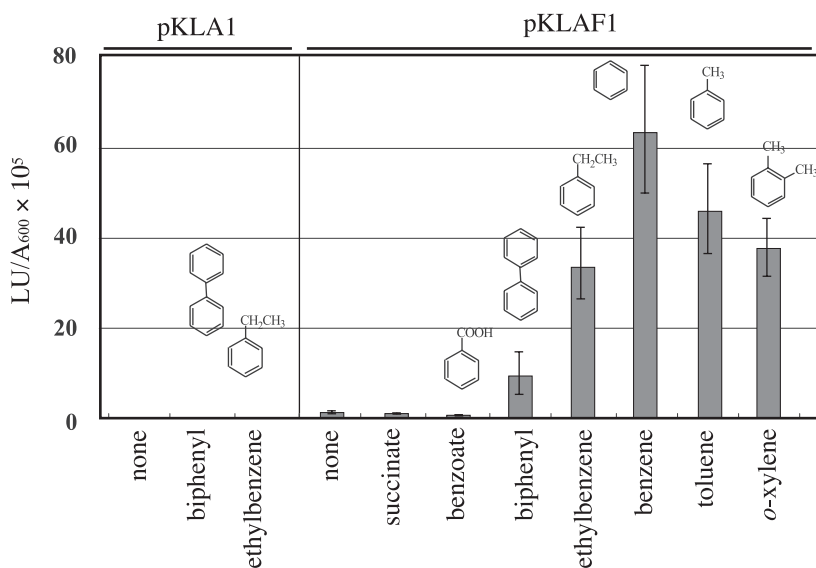
Isolation of a fragment activating transcription from P_{bphA1} . Regulatory genes located adjacent to the corresponding degradation enzyme genes have been reported frequently (15, 16, 33). The 8.8-kb HindIII fragment containing the region downstream of *bphB* was cloned in the KpnI site of pKLA1 to obtain pKA851 (Fig. 3A). When pKA851 was introduced into a rhodococcal host strain devoid of *bph* genes, *R. erythropolis* IAM1399, induction of P_{bphA1} was observed with a broad range of aromatic compounds, including biphenyl, ethylbenzene, benzene, toluene, xylenes, cumene, *p*-cymene, and chlorinated benzenes. Induction was not observed with benzoate, succinate, and *p*-chlorobiphenyl (Fig. 3C). These results suggest that this 8.8-kb HindIII fragment contains a regulatory gene(s) that promotes induced activation of P_{bphA1} by a variety of aromatic compounds. In the absence of aromatic compounds, the cells carrying pKA851 exhibited low luciferase activity which was higher than the luciferase activity of the cells carrying pKLA1, suggesting that the basal activation of P_{bphA1} is promoted by the regulatory gene(s) in the 8.8-kb HindIII fragment.

To determine the transcription initiation site of the *bphA1* gene, we performed primer extension analysis. Total RNAs were prepared from IAM1399 cells harboring pKA851 grown in LB medium, on biphenyl, or on ethylbenzene. The primer extension products were observed with RNA from the cells grown on biphenyl or ethylbenzene but not with RNA from the cells grown in LB medium. A single major product, designated P1 (Fig. 4), was found, and this product represented transcription starting 156 bp upstream from the ATG initiation codon. Three minor products, P2 to P4 (Fig. 4), were also detected, and these products had transcription starts 66, 50, and 39 bp upstream from the initiation codon, respectively. Total RNA from the IAM1399 cells harboring pKLA1 gave no product. Possible promoter consensus sequences of *E. coli*, *Bacillus*, and *Streptomyces coelicolor* were not identified at the appropriate

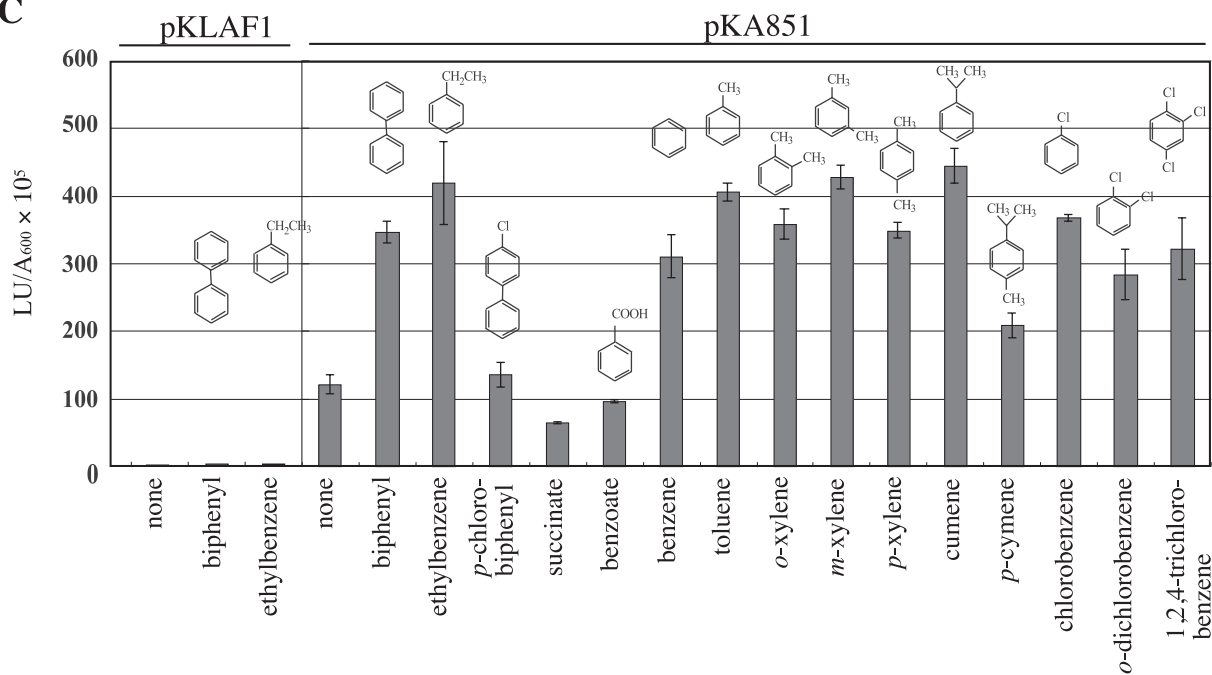
A



B



C



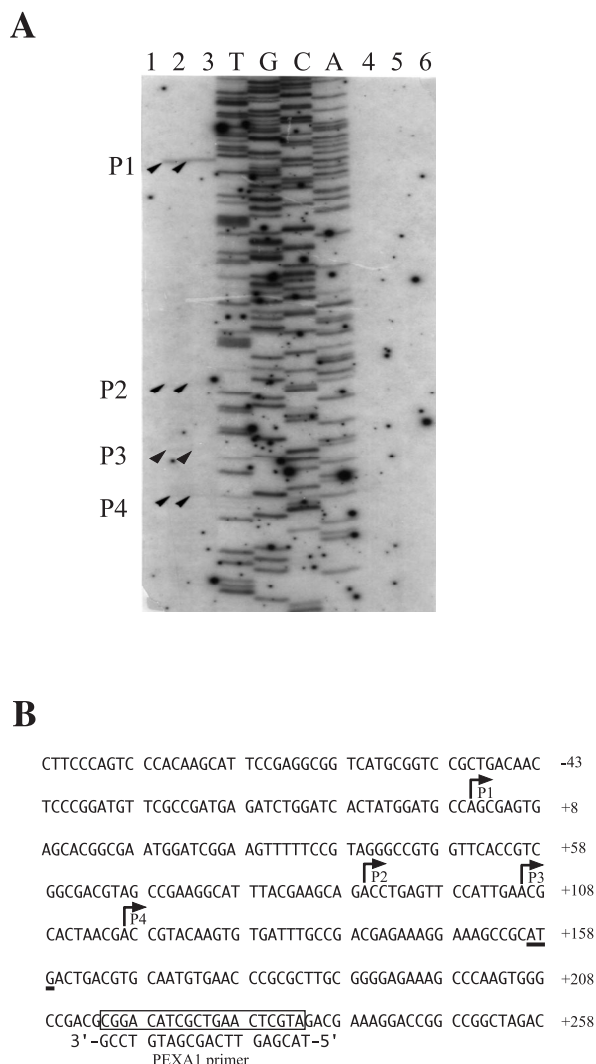


FIG. 4. Transcription start site of *bphA1*. (A) Primer extension analysis of *bphA1*. Total RNA was isolated from IAM1399 cells harboring pKA851 (lanes 1, 2, and 3) or pKLA1 (lanes 4, 5, and 6) grown on LB medium (lanes 1 and 4), biphenyl (lanes 2 and 5), or ethylbenzene (lanes 3 and 6). Lanes T, G, C, and A, sequencing ladder for pKLA1 obtained by using the PEXA1 primer. The primer extension products are indicated by arrowheads and are designated P1, P2, P3, and P4. (B) Nucleotide sequence of the upstream region of *bphA1*. The deduced transcriptional start sites for P1, P2, P3, and P4 are indicated by bent arrows. The open box indicates the position of the PEXA1 primer, whose sequence is indicated under the box. The start codon of *bphA1* is underlined. Nucleotide numbers were assigned by using the transcriptional start site of P1 (position 1) as the reference point.

FIG. 3. Transcriptional activation of *bphA1* promoter. (A) Physical maps of pKLA1 and pKA851. pKLA1 was constructed by inserting the 1.4-kb XhoI-BamHI *bphA1* promoter fragment into a promoter probe vector, pKLA1. The 8.8-kb HindIII fragment containing the region downstream of *bphB* was cloned in the KpnI site of pKLA1 to construct pKA851. The restriction sites in parentheses are the sites that were used to generate fragments and were lost by blunt-end formation prior to ligation. (B) Luciferase activity of *Rhodococcus* sp. strain RHA1 harboring pKLA1. Cells were grown in 0.2× LB medium in the absence or presence of the substrates. The chemical structures of the substrates are shown. The data are means ± standard deviations from at least three determinations. The luciferase activities of RHA1 cells harboring a promoter probe vector, pKLA1, were all less than 0.1 × 10⁵ light units (LU) per A₆₀₀ unit (see Materials and Methods). (C) Luciferase activity of *R. erythropolis* IAM1399 harboring pKLA1 or pKA851. Cells were grown in 0.2× LB medium in the absence or presence of substrates. The data are means ± standard deviations from at least three determinations.

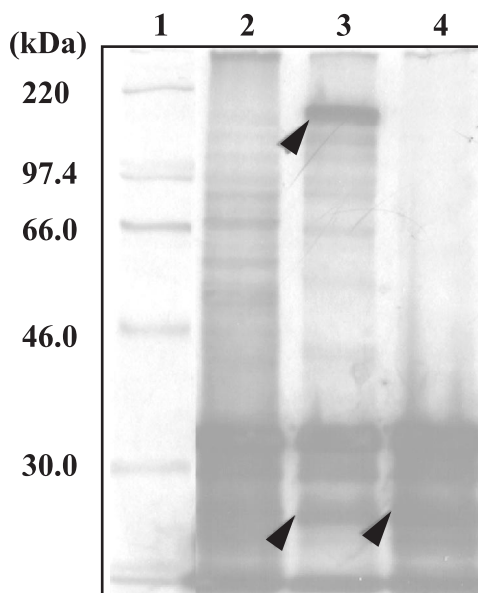


FIG. 5. In vitro expression of the *bphS* and *bphT* genes of *Rhodococcus* sp. strain RHA1. The *bphST* and *bphT* genes in pBAB62 and pBAB14, respectively, were expressed in the *E. coli* S30 extract system containing [³⁵S]methionine. Labeled polypeptides were separated by SDS-PAGE. The expected *bphS* and *bphT* products are indicated by arrowheads. Lane 1, ¹⁴C-labeled molecular weight marker (Pharmacia); lanes 2, 3, and 4, gene products of pBSL (vector control), pBAB62, and pBAB14, respectively.

positions for the transcription start sites (P1 to P4). The P2 to P4 minor products might have been generated by 5' end processing of the major P1 transcript.

Sequence analysis of the 8.8-kb HindIII fragment. The nucleotide sequence of the 8.8-kb HindIII fragment was determined. Eight open reading frames (ORFs) were found in the 8,801-bp HindIII fragment in addition to the carboxyl-terminal portion of *bphB*. The deduced amino acid sequences encoded by ORF 1 (1,315 to 6,108 bp) and ORF 3 (6,108 to 6,734 bp) showed significant identity with the sequences encoded by putative two-component regulatory genes, *bpdS* (55%) and *bpdT* (63%), respectively, of a rhodococcal PCB degrader, *Rhodococcus* sp. strain M5 (15). ORF 5 (8,296 to 6,761 bp) showed identity with benzoate coenzyme A ligase of *Thauera aromatica* (21%). The remaining ORFs overlapped these ORFs, and the sequences which they encoded exhibited no homology with known proteins. Thus, ORF 1 and ORF 3 were designated *bphS* and *bphT*, respectively. The *bphS* gene, which starts 627 bp downstream from the *bphB* stop codon, was 4,794 bp long, and its TGA termination codon overlapped the ATG start codon of *bphT*, which was 627 bp long. The *bphS* and *bphT*

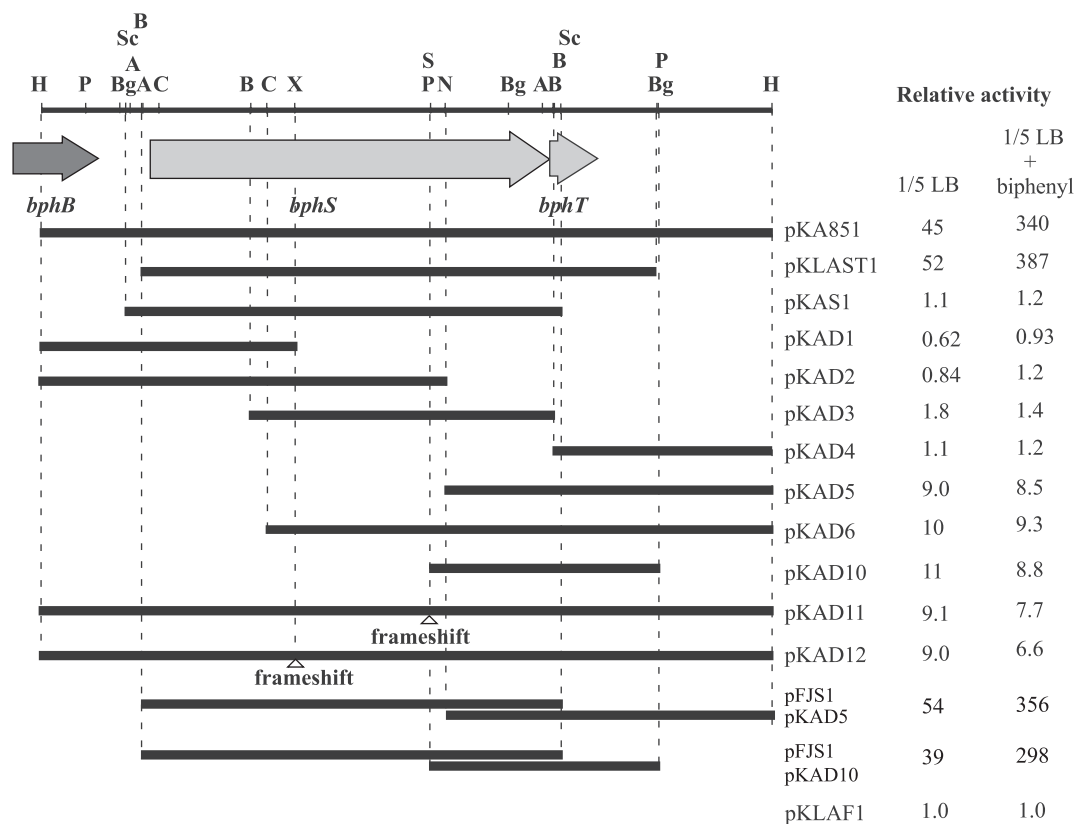


FIG. 6. Deletion analysis of the 8.8-kb HindIII DNA fragment containing the *bphST* genes. The segments represented by solid bars were inserted into the KpnI site of pKLAf1. The IAM1399 cells harboring the plasmids were grown in 0.2× LB medium (1/5 LB) in the presence or absence of biphenyl and were subjected to the luciferase assay. The relative luciferase activities conferred by each construct in comparison to the activities of the cells harboring pKLAf1 were estimated and are indicated on the right. pKLAf1 is a reporter plasmid that does not contain any *bphST* segment. The open triangles indicate the positions of frameshift mutations generated by filling in of restriction fragment termini by T4 polymerase. Restriction enzyme site abbreviations: A, ApaI; B, BamHI; Bg, BglII; C, ClaI; H, HindIII; N, NotI; P, PstI; S, Sse8387I; Sc, SacI; X, XmaI.

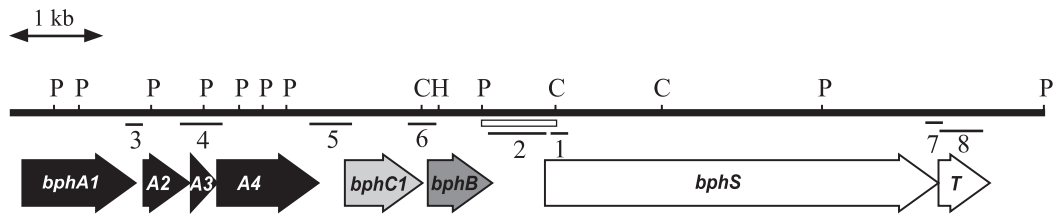
genes encoded polypeptides containing 1,598 and 209 amino acids, whose molecular masses were calculated to be 173.5 and 22.9 kDa, respectively.

To identify the gene products, the whole-cell lysates of *E. coli* cells containing pBAB62 and pBAB14, which had inserts of the *bphST* and *bphT* genes in the vector pBSL, respectively, were prepared and subjected to SDS-PAGE as described in Materials and Methods. No induced proteins corresponding to the *bphS* and *bphT* gene products were observed. We also examined expression of the *bphS* and *bphT* genes under control of the T7 promoter of the pET21 vector in *E. coli* BL21, but the results were negative. We then employed an in vitro transcription-translation assay system. Plasmids pBAB62 and pBAB14 were used as templates for the *E. coli* S30 extract system (Fig. 5). Two polypeptide bands, at 175 and 25 kDa, were observed with pBAB62, and these molecular masses were in good agreement with those deduced from amino acid sequences of BphS (173.5 kDa) and BphT (22.9 kDa). The 25-kDa polypeptide band was also observed with pBAB14.

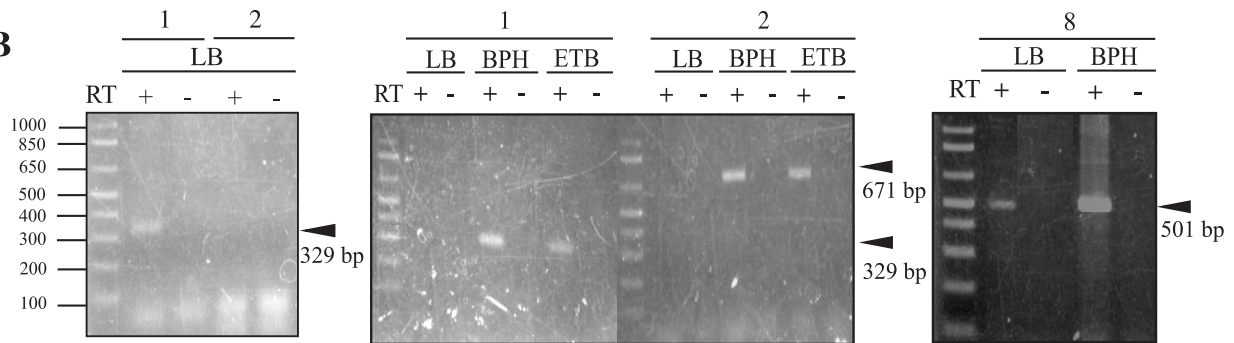
Deletion analysis of the 8.8-kb HindIII fragment. To investigate the functional involvement of the products of *bphST* in the activation of P_{bphA1} , various subclones of the 8.8-kb HindIII fragment shown in Fig. 6 were constructed by inserting each fragment into the KpnI site of pKLAf1. The resultant

plasmids were transformed into IAM1399 by electroporation, and the luciferase activity of each transformant was measured in the presence and absence of biphenyl (Fig. 6). In addition to pKA851 containing the entire 8.8-kb HindIII fragment, pKLAST1 containing just the *bphS* and *bphT* genes showed basal activation and biphenyl-induced activation of P_{bphA1} compared with the reporter plasmid, pKLAf1. Clones not containing the whole *bphT* gene (pKAS1, pKAD1, pKAD2, pKAD3, and pKAD4) showed no activation of P_{bphA1} even in the presence of biphenyl. One of these clones, pKAS1, contained the intact *bphS* gene, suggesting that the *bphS* gene product has no direct effect on P_{bphA1} . Clones containing only the entire *bphT* gene (pKAD5, pKAD6, and pKAD10) showed basal activation of P_{bphA1} . The clones containing a frameshift mutation of *bphS* and an intact *bphT* gene (pKAD11 and pKAD12) also exhibited basal activation of P_{bphA1} . The basal activation of P_{bphA1} observed with pKAD5 or pKAD10 was elevated by introduction of pFJS1 carrying an intact *bphS* gene. These results suggest that *bphT* has fundamental P_{bphA1} activation activity, which is elevated by *bphS*, and that both *bphS* and *bphT* are essential for the induced activation by biphenyl. Thus, BphS and BphT seem to be a sensor kinase and a response regulator, respectively, of a two-component regula-

A



B



C

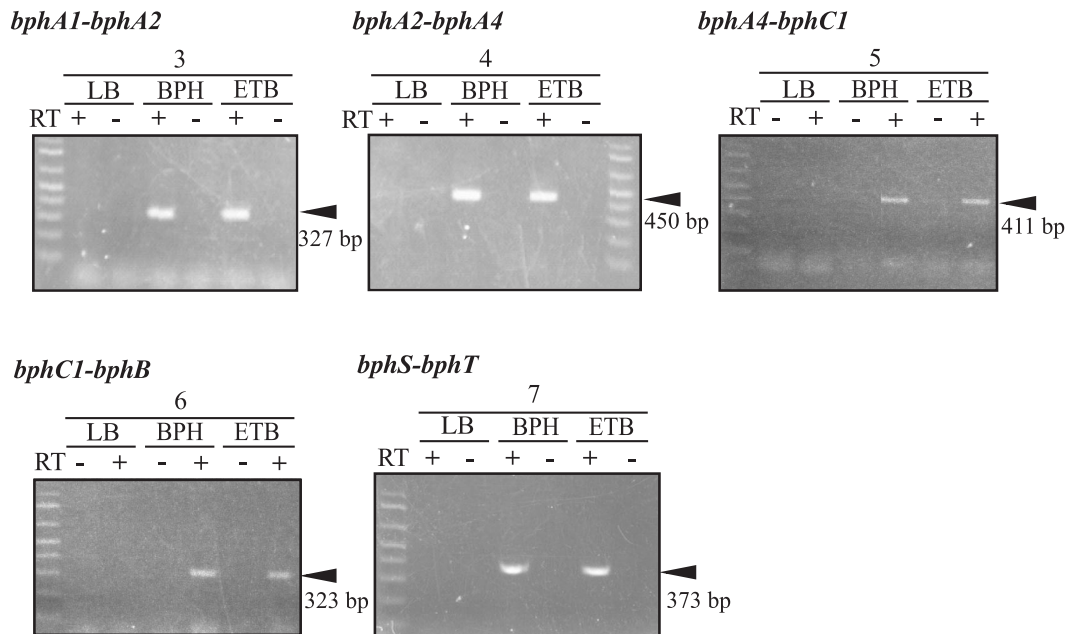
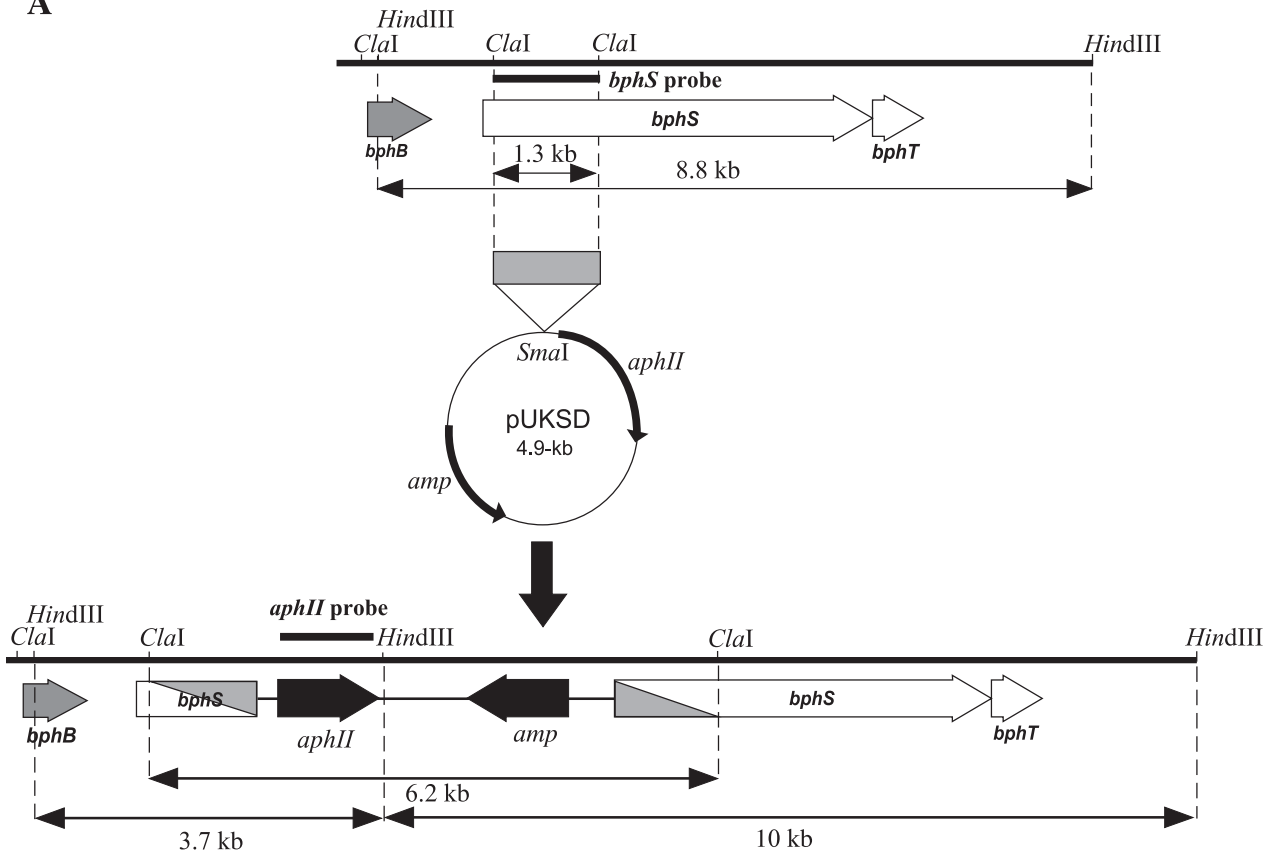
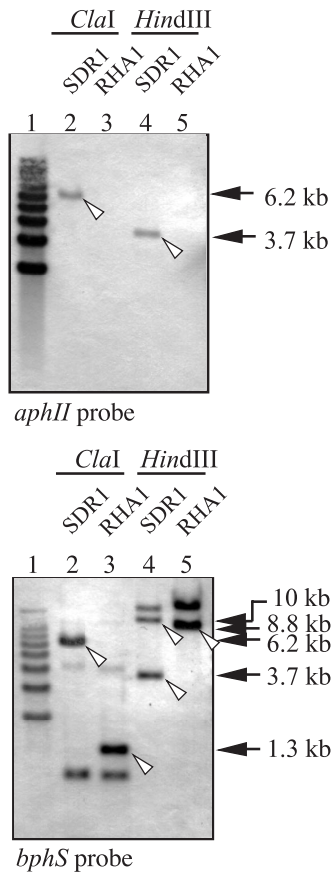


FIG. 7. Transcription of the *bphS* gene in RHA1. (A) Physical map of the region from *bphA1* to *bphT*. The open box below the map indicates the fragment used to construct reporter plasmids pKLASF and pKLASR. The lines below the map indicate intergenic or internal segments of *bph* genes that were expected to be amplified by RT-PCR. The numbers below the lines indicate the PCR primer sets shown in Table 2. Restriction enzyme site abbreviations: P, PstI; C, ClaI; H, HindIII. (B) *bphS* and *bphT* gene transcripts in RHA1. Total RNAs from RHA1 cells grown on LB medium, on biphenyl (BPH), or on ethylbenzene (ETB) were reverse transcribed under the intensive conditions (left and right panels) or the usual conditions (center panel). The reverse transcripts were subjected to PCR amplification by using the primer sets indicated by the numbers above the panels, which correspond to the numbers in panel A and Table 2. RNA samples were concurrently analyzed in PCR mixtures with (+) and without (-) reverse transcriptase (RT) to verify the absence of total DNA. The position and size of each PCR product are indicated by an arrowhead on the right. (C) Intergenic RT-PCR products of the *bphA1A2A3A4C1BST* genes in RHA1. Total RNAs from RHA1 were reverse transcribed under the usual conditions, amplified, and analyzed as described above. An arrowhead indicates the position and size of each PCR product.

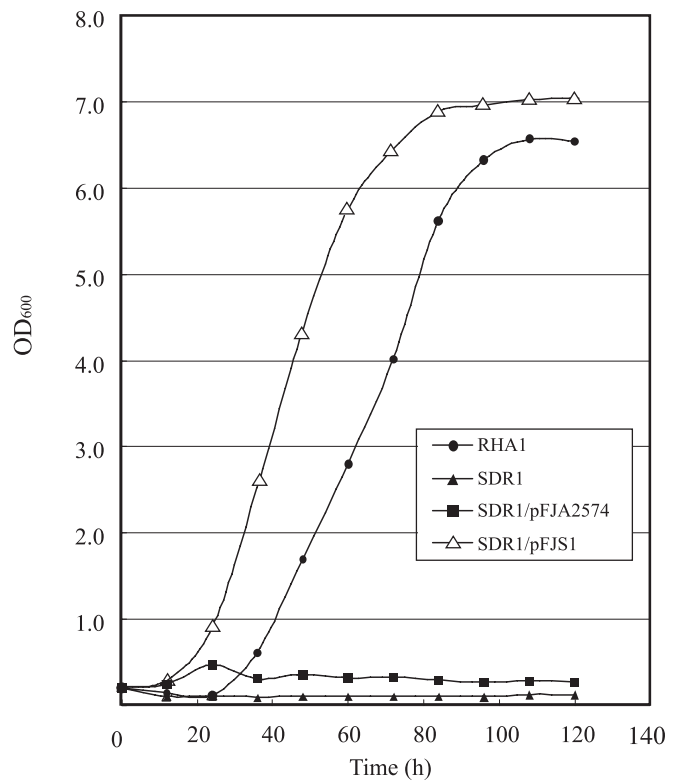
A



B



C



tory system governing the induced transcription activation of P_{bphA1} .

Constitutive and induced transcription of *bphS*. To promote inducible activation of degradation genes, the *bphST* genes need to be transcribed constitutively in the absence of an inducer. The total RNA from RHA1 cells grown on LB medium was subjected to RT-PCR analysis by using the primer set designed to amplify the internal segment of *bphS*. No amplification product was observed after RT-PCR performed under the usual conditions (Fig. 7B, center panel). An amplification product of the expected size (329 bp) was obtained when RT-PCR was performed under the intensive conditions (Fig. 7B, left panel), which generated more PCR product and provided higher sensitivity than the usual conditions. The primer set designed to amplify the internal segment of *bphT* also gave an amplification product corresponding to the expected size (501 bp) under the intensive conditions (Fig. 7B, right panel). The primer set designed to amplify the intergenic segment between *bphB* and *bphS* (671 bp) gave no product even under the intensive conditions (Fig. 7B, left panel). Then the promoter activity of the adjacent region upstream of *bphS* was examined. The PstI-ClaI fragment containing the region upstream of *bphS* (Fig. 7A) was inserted into the Sall site of pKLA1 to form pKLASF, which was introduced into RHA1. Compared with the activity observed with cells containing pKLA1, about 15-fold-greater luciferase activity was observed in RHA1 cells containing pKLASF even in the absence of biphenyl and ethylbenzene. When the fragment upstream of *bphS* in pKLASF was reversed to create pKLASR, no increase in luciferase activity was observed in RHA1 cells carrying pKLASR. These results indicate that the *bphST* genes are transcribed constitutively from a promoter in the adjacent region upstream of *bphS* in RHA1.

When RT-PCR analysis of the internal region of *bphS* was performed with RNA from the cells grown on biphenyl or ethylbenzene, an intense PCR product was observed; in contrast, the cells grown in LB medium gave no product (Fig. 7B, center panel). In this analysis, the primers for an intergenic segment between *bphB* and *bphS* also gave an intense product. These results suggest that transcription occurs from another upstream promoter, which is thought to be located within or upstream from *bphB*. As no terminator sequence was identified by nucleotide sequence analysis between *bphB* and *bphS*, it is possible to deduce that there was induced transcription of *bphS* from P_{bphA1} . Thus, transcription of genes from *bphA1* to *bphT* was examined. The total RNAs prepared from RHA1 cells were subjected to RT-PCR analysis by using the primer sets expected to amplify intergenic segments 2 to 7, as shown in

Fig. 7A. All the primer sets gave the corresponding amplification products of the expected sizes with the RNA prepared from the cells grown on biphenyl or ethylbenzene (Fig. 7C). None of the amplification products were detected with RNA from the cells grown in LB medium, indicating that transcription of all the *bphA1A2A3A4C1BST* genes was induced simultaneously by biphenyl and ethylbenzene.

Disruption of the *bphS* gene in RHA1. To determine whether the *bphS* gene is really responsible for biphenyl catabolism in RHA1, the *bphS* gene was inactivated with a disruption plasmid, pUKSD, by homologous recombination, as shown in Fig. 8A. Southern hybridization analysis was performed with *aphII* and *bphS* probes to confirm the expected arrangement of disrupted *bphS* sequences (Fig. 8B). In a kanamycin-resistant transformant, SDR1, both the *aphII* and *bphS* probes hybridized to a 6.2-kb ClaI fragment, which was 4.9 kb larger than the 1.3-kb RHA1 *bphS* fragment. The *aphII* probe hybridized to a 3.7-kb HindIII fragment containing *aphII*. The *bphS* probe hybridized to 3.7- and 10-kb HindIII fragments of SDR1, as pUKSD contains a HindIII site. The sum of the sizes of the hybridized HindIII fragments was 13.7 kb, and this value was 4.9 kb larger than the size of the RHA1 *bphS* fragment (8.8 kb). These results indicate that the entire 4.9-kb pUKSD segment was integrated into the *bphS* gene, as expected. Apart from the expected signals for *bphS* fragments, we found additional signals which suggested the existence of a *bphS* homologue in RHA1. SDR1, however, did not grow on biphenyl as a sole carbon source, indicating that the *bphS* gene alone is essential for growth of RHA1 on biphenyl (Fig. 8C).

To complement the *bphS* gene defect of SDR1, pFJS1 containing an intact *bphS* gene was constructed and introduced into SDR1. SDR1 carrying pFJS1 grew on biphenyl better than RHA1 grew on biphenyl (Fig. 8C). Thus, introduction of an intact *bphS* gene fully restored the growth of SDR1 on biphenyl. The excessive expression of *bphS* in a multicopy plasmid seemed to cause higher expression of *bphA1A2A3C1B*, resulting in better growth on biphenyl. These results suggest that the *bphS* gene alone is responsible for the growth of RHA1 on biphenyl.

DISCUSSION

We found in the present study that the *bphS* and *bphT* genes are likely to encode a two-component regulatory system responsible for transcriptional induction of the *bphA1* promoter, P_{bphA1} , by aromatic compounds, including biphenyl, based on the following results. (i) BphS and BphT exhibited identity and shared conserved residues with previously characterized sensor

FIG. 8. *bphS* gene disruption in RHA1. (A) Strategy for *bphS* gene disruption. Disruption was accomplished by a single crossover between the native *bphS* gene (top) and the 1.3-kb internal fragment of *bphS* in pUKSD (middle). The possible region of recombination in the disruption derivative (bottom) is shaded diagonally. The positions of the *bphS* and *aphII* probes used in Southern hybridization are indicated by thick lines above *bphS* (top) and *aphII* (bottom). The sizes of ClaI and HindIII fragments containing the *bphS* sequence are indicated below the ORF maps (top and bottom). (B) Southern hybridization analysis of *bphS* disruption mutant strain SDR1 performed with the *aphII* (upper panel) and *bphS* (lower panel) probes. Lane 1, 1-kb ladder marker; lane 2, SDR1 total DNA digested with ClaI; lane 3, RHA1 total DNA digested with ClaI; lane 4, SDR1 total DNA digested with HindIII; lane 5, RHA1 total DNA digested with HindIII. The position and size of each signal derived from the bona fide *bphS* sequence are indicated by an open arrowhead and an arrow on the right, respectively. (C) Growth on biphenyl of SDR1 and *bphS* gene-containing SDR1. RHA1, SDR1, SDR1 carrying the vector pFAJ2574, and SDR1 carrying the *bphS* plasmid pFJS1 were grown in W minimal medium containing 0.2% biphenyl. Growth was measured by determining the optical density at 600 nm (OD_{600}). The data are averages based on triplicate experiments.

kinases and response regulators of two-component regulatory systems. (ii) *bphS* and *bphT* promoted transcriptional induction of P_{bphA1} in a host strain, *R. erythropolis* IAM1399. (iii) Both the *bphS* and *bphT* products were required for the transcriptional induction of P_{bphA1} in IAM1399. (iv) *bphT* alone was responsible for basal transcription of P_{bphA1} in IAM1399. (v) In the absence of *bphT*, *bphS* did not activate transcription of P_{bphA1} at all. (vi) In the presence of *bphT*, *bphS* was responsible for elevated basal transcription of P_{bphA1} and was required for the induced activation of P_{bphA1} transcription. In *Pseudomonas* sp. strain Y2, similar basal activation of a responsible promoter of *styAB* in the absence of an inducer by the two-component regulatory system encoded by *stySR* was reported previously (36). This activation may be due to both basal activation by the *styR* product and elevation by the *styS* product, as observed with RHA1 *bphST*.

Because these results were obtained with *R. erythropolis* IAM1399, they do not necessarily indicate that *bphS* and *bphT* are indispensably involved in the induction of P_{bphA1} by biphenyl in RHA1 and in the growth of RHA1 on biphenyl. Thus, we disrupted the *bphS* gene and complemented the *bphS* mutation. The results obtained in this study indicated that at least *bphS* is indispensably responsible for P_{bphA1} induction by biphenyl and the growth of RHA1 on biphenyl. A Southern hybridization experiment to confirm disruption of the *bphS* gene indicated that there is a *bphS* homolog in RHA1. This *bphS* homolog seems not to be involved in induction of the *bphA1A2A3A4C1B* genes by biphenyl, as *bphS* gene disruption was found to result in a stringent growth deficiency on biphenyl. Although all attempts to disrupt the *bphT* gene failed, the following results suggest that *bphT* plays at least a partial role in P_{bphA1} induction by biphenyl. (i) *bphT* was required for P_{bphA1} induction by biphenyl in IAM1399. (ii) *bphT* was expressed transcriptionally in RHA1. (iii) *bphT* was located next to *bphS* in a manner implying translational coupling and in the adjacent region downstream of *bphA1A2A3A4C1B*.

Several two-component regulatory systems involved in the degradation of aromatic compounds have been described previously. These systems include BpdST of PCB-degrading *Rhodococcus* sp. strain M5 (15), TodST of the toluene degraders *Pseudomonas putida* F1 (16) and *P. putida* DOT-T1 (22), TutCB and TutC1B1 of the anaerobic toluene degradation pathway of *T. aromatica* T1 (4), StySR of the styrene degradation pathway of *Pseudomonas* sp. strain Y2 (36) and *Pseudomonas fluorescens* ST (17), TdiSR of the anaerobic toluene degradation pathway of *Azoarcus* sp. strain T (1) and *T. aromatica* K172 (18), and TmoST of a toluene degrader, *Pseudomonas mendocina* KR1 (27). Except for BpdST of M5, these two-component systems are responsible for the degradation of monocyclic aromatic compounds in gram-negative bacteria. With a focus on the transcriptional regulatory systems for biphenyl and PCB degradation pathways, several systems have been described. The systems in gram-negative bacteria, including *R. eutropha* A5 (23), *Pseudomonas* sp. strain KKS102 (24), *P. pseudoalcaligenes* KF707 (37), and *Burkholderia* sp. strain LB400 (3), belong to the GntR family. In contrast, the systems in gram-positive bacteria, including strain RHA1 and *Rhodococcus* sp. strain M5, are two-component systems. These results suggest that there was independent evolution of regulatory genes in gram-positive and gram-negative bacteria.

Involvement of a two-component regulatory system in induction of the biphenyl degradation pathway of a rhodococcal PCB degrader has been suggested previously for *Rhodococcus* sp. strain M5, as determined by nucleotide sequence analysis and *bpdS* gene disruption (15). In this study, we found the two-component regulatory system encoded by RHA1 *bphST* not only by nucleotide sequence analysis but also by deletion and frameshift mutation analysis with a reporter assay in IAM1399. The involvement of *bphS* in growth on biphenyl was confirmed by a complementation experiment, which excluded the polar effect of gene disruption. Thus, this study illustrated the functional roles and induction characteristics of *bphST* gene products and the exact involvement of *bphS* in growth on biphenyl.

The amino terminus of BphS was found to contain a domain that is similar to the serine/threonine kinases (Fig. 9), as well as BpdS (15). We compared the BphS and BpdS amino-terminal domains with PKA-C α , which is the best-characterized serine/threonine kinase to date (8). BphS has most of the key residues that are highly conserved in serine/threonine kinases. T156 seems to be the counterpart of PKA-C α T196, which was found to be the phosphorylation site. BphS lacks counterparts of DFG in subdomain VII and RD in the consensus sequence H/YRDLKXXN in subdomain VIB. Because the D residue in the consensus sequence H/YRDLKXXN was estimated to be a catalytic base, which is essential for the catalytic reaction (8), the amino-terminal domain of BphS may not function as a serine/threonine kinase. The carboxyl-terminal region of BphS shares residues with the HPK7 subfamily (7) of sensor kinase proteins such as DegS, UhpB, and VsrA, as pointed out for BpdS (15). It has the conserved residues in the H-box, N-box, D-box, and G-box of histidine kinases, as proposed by Stock et al. (34, 35). H1411 in the H-box and the G-box from residue G1563 to residue V1594 are thought to be an autophosphorylation site and a nucleotide-binding site, respectively.

BphT shares residues with the response regulator proteins of two-component regulatory systems, such as DegU, UhpA, and VsrD, as well as BpdT (15). Among the residues that are conserved, D8, D9, and D54 appear to correspond to the residues which have been proposed to constitute an acid pocket for phosphorylation (26). D54 may serve as the main phosphorylation site, and K104 is a seemingly invariant residue that has been proposed to play a key role in the response activity (26).

In the presence of the BphST regulatory system in heterologous host strain IAM1399, P_{bphA1} was induced by a variety of aromatic compounds, including biphenyl, ethylbenzene, benzene, toluene, xylenes, cumene, *p*-cymene, and chlorinated benzenes, suggesting that the BphST regulatory system has a significantly broad spectrum of inducers. The inducer spectrum of the *todX* promoter in *P. putida* DOT-T1 was described without characterization of the responsible regulatory system (22). Using a heterologous host strain, we examined the exact BphST-dependent induction, which is expected to reflect the exact features of the BphST regulatory system. However, it seems to be impossible to compare the inducing activities of substrate compounds because the solubility in the medium and the permeability of inducers through the cell membrane are estimated to be different. P_{bphA1} was not induced by succinate, benzoate, and *p*-chlorobiphenyl. The inability of *p*-chlorobi-

		I	II	III		
BphS	5	TVRVLRSQSG	ITTTL--ALS	GETRERVVVR	RVDLSMTFPW S-WHWLEGE	51
BpdS	5	TVKILRSDSPG	ITTTL--VVS	GEDRERVVLR	RLDLGRTWLW S-RRWLEDEV	51
PKA-C α	44	FERIKLTGTG	SFGRVMLVKH	KETGNHYAMK	ILDQKQVVKL KQIEHTLNEK	93
Jnk3s	64	YQNLKPIGSG	AQGIVCAAYD	AVLDRNVAIK	KLSRPFQNK- THAKRAYRE	112
Ste20	620	YANLVKIGQG	ASGGVYTAYE	IGTNVSVAIK	QMNLEKQPK- KEL--IINEI	666
		IV	V			
BphS	52	EAMRRARLPH	VIPTQIAHKG	PDHVDLVRP-	FIAGLDIREW SAQESPQSL-	99
BpdS	52	EAIRRARLSH	VIAPTIVHHG	PDHIDLVRP-	FIEGMDVREW FASHPTRSF-	99
PKA-C α	94	RILQAVNFPF	LVKLEFSFKD	NSNLVMME-	YVPGGEMFESH LRRIGR--FS	140
Jnk3s	113	VLMKCVNHKN	IISLLNVFTP	QKTLLEEFQDV	YVMEELMDAN LCQVIQMLD	162
Ste20	667	LVMKGSKHPN	IVNFIDSYVL	KGDLWVIME-	YMEGSLTLDV VTHC-I--LT	712
		VIA	VIB	VII		
BphS	100	DVQLQMC-N	LFYALARLHR	MGIAHGGVVKP	ANITMLA-EGT NQLVLLD--A	145
BpdS	100	AEQLRIMA-T	LFHALACLHR	LGIAHGGVTP	ANIVLT-ENG -ELVLLD--A	144
PKA-C α	141	EPHARFYAAQ	IVLTFEYLHS	LDLIYRDLKP	ENLLIDQQGY IQVTDGFAK	190
Jnk3s	163	HERMSYLLYQ	MLCGIKHLHS	AGIIHRDLKP	SNEVVKSDCT LKILDFGL-A	211
Ste20	713	EGQIGAVCRE	TLSGLEFLHS	KGVLHRDIKS	DNILLSMEGD IKLTDGFCFA	762
				*****	*	***
		VIII	IX			
BphS	146	---SVTRTQ	LAAVTHPVEG	PEGRYLLPES	PGLAH-PTAG FTADIFAAGW	190
BpdS	145	---GVTRVQ	LAA-TNDQPT	TSSIGADRGD	PSRNN-GTGG FSADLFGAGL	188
PKA-C α	191	RVKG---RTW	TLCGTPPELA	PEIILSKGYN	KAVDWWALGV LIYEMAAAGYP	237
Jnk3s	212	RTAGTSFMMT	PYVVTRYRYRA	PEVILGMGYK	ENVDIWSVGC IMGEMVR-HK	260
Ste20	763	QINELNLKRT	TMVCTPYWMA	PEVVSKEYG	PKVDIWSLGI MIEMIEGEP	812
			*			

FIG. 9. Comparative features of the amino terminus of BphS. The boxes indicate invariant residues in sequences. The amino terminus of BphS is aligned with those of protein kinases, including human cAMP-dependent protein kinase catalytic subunit PKA-C α (National Center for Biotechnology Information protein database accession number P17621), *Saccharomyces cerevisiae* Ste20 (AAA35039), human mitogen-activated protein kinase JNK3 (Jnk3s) (U76020), and BpdS (AAB52543). The roman numerals indicate the positions of conserved subdomains in eukaryotic serine/threonine kinases (8). The asterisks indicate the residues mentioned in the text.

phenyl to induce P_{bphA1} agrees with the requirement of biphenyl for cometabolic PCB degradation as an inducer of P_{bphA1} .

RT-PCR analysis of the *bphA1A2A3A4C1B* and *bphST* genes indicated that the induced transcription by biphenyl continues from gene to gene. These results did not exclude transcription termination within a gene. Our results imply the operonic structure of *bphA1A2A3A4C1BST* but do not exclude the possibility that some unidentified promoter(s) other than P_{bphA1} , which is located in the region from *bphA1* to *bphB*, is also responsible for the induction of *bphST* transcription by biphenyl. Like expression in RHA1, it has been suggested that expression of the *bpdC1C2BADE* and *bpdST* genes in *Rhodococcus* sp. strain M5 is induced by biphenyl (15). In the presence of biphenyl transcription seems to terminate between *bpdE* and *bpdS* in M5, suggesting that there is an inducible promoter for *bpdS* in the adjacent upstream region. We found that in RHA1 there is a constitutive promoter in the adjacent upstream region of *bphS* (P_{bphS}), which seems to be responsible for the transcription of *bphS* in the absence of biphenyl. In the presence of biphenyl, RT-PCR analysis of the region between *bphB* and *bphS* suggested that the induced transcription of *bphB* extends to *bphS* to a considerable extent. Thus, an inducible promoter for *bphS* is thought to reside in the distant upstream region of *bphS*. Based on the results of the RT-PCR analysis of the intergenic regions of *bphA1* to *bphS*, P_{bphA1}

seems to be a good candidate for this inducible promoter for *bphS*. These results led us to propose the following probable scheme of induction by *bphST*. In the absence of biphenyl, *bphST* genes are constitutively transcribed from the adjacent P_{bphS} promoter at the basal level. In the presence of biphenyl, biphenyl activates the *bphS* product (BphS), which then activates the *bphT* product (BphT) by phosphorylation. The activated BphT promotes transcription initiation from P_{bphA1} and induces expression of the *bphA1A2A3A4C1B* and *bphST* genes.

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