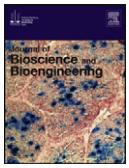

Journal of Bioscience and Bioengineering
VOL. 113 No. 3, 279–285, 2012

ELSEVIER

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The 24-bp consensus sequence responsible for regulation of the BphS1T1 two-component system in a hybrid promoter

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Received 2 September 2011; accepted 27 October 2011
Available online 1 December 2011

Rhodococcus jostii **RHA1 degrades polychlorinated biphenyls (PCBs) by cometabolism with biphenyl. The *bphS1T1*-coding two-component system, which is composed of a sensor kinase, BphS1, and a response regulator, BphT1, activates the transcription of biphenyl/PCB degradation genes from the five promoters of *bphAa*, *etbAa1*, *etbAa2*, *etbAd*, and *etbD1* in the presence of aromatics, such as biphenyl and ethylbenzene. The transcription start sites of *etbAd* and *etbD1* were determined and the results indicated that the 18-bp consensus sequence is shared by all five promoters at the equivalent position from their transcriptional start sites. To investigate the involvement of the 18-bp consensus sequence in the regulation of BphS1T1, a hybrid promoter was constructed by connecting the 18-bp consensus sequence of *bphAa* promoter to a portion of the benzoate dioxygenase gene promoter, which is not under the control of BphS1T1. The ethylbenzene-dependent induction of the hybrid promoter by BphS1T1 was not observed. Recently, a 24-bp consensus sequence that included the 18-bp consensus sequence of the *bphAa* promoter was identified in the regions conserved among RHA1 and other rhodococcal degraders. When the 24-bp consensus sequence was employed instead, both BphS1T1-dependent basal activation and ethylbenzene-dependent induction of the hybrid promoter were observed. Mutations in the six extra residues outside the 18-bp sequence in the 24-bp consensus sequence, affected not only ethylbenzene-dependent induction but also BphS1T1-dependent basal activation. The outstanding conservation of the 24-bp consensus sequence was confirmed by multiple sequence alignment. These results indicate that the 24-bp consensus sequence is really responsible for the regulation of BphS1T1.**

[**Key words:** Two-component system; Hybrid promoter; Transcriptional activation; *Rhodococcus*]

Rhodococcus jostii RHA1 is a gram-positive bacterium that possesses an exceptional capacity to aerobically degrade polychlorinated biphenyls (PCBs) by the biphenyl degradation enzyme pathway (1–6), and it is of interest for a range of bioremediation techniques. Degradation enzyme genes for the upper biphenyl pathway reside in two linear plasmids, pRHL1 (1123 kb) and pRHL2 (443 kb) (7). They are distributed in pRHL1 as gene clusters *bphAaAbAcAdC1B1* and *etbD1*, and in pRHL2 as *etbAa1Ab1CbphD1*, *etbAa2Ab2AcD2*, and *etbAdbphB2*. The transcription of these genes from the promoters for *bphAa*, *etbAa1*, *etbAa2*, *etbAd*, and *etbD1* (*bphAap*, *etbAa1p*, *etbAa2p*, *etbAdp*, and *etbD1p*, respectively) are simultaneously induced in the presence of aromatic compounds, including biphenyl and ethylbenzene (8). These five promoters are under the control of dual two-component systems, which are coded by *bphS1T1* and *bphS2T2*, located in pRHL1 and pRHL2, respectively (9,10). Two-component systems are employed by a number of bacteria and certain higher organisms, and they conduct the signal transduction that regulates cellular functions in response to environmental stimuli (11,12). The typical two-component system consists of a sensor protein-histidine kinase (HK) and a response regulator (RR). The deduced amino acid sequences of a *bphS1* product (BphS1) and a *bphS2* product (BphS2) are nearly identical (92%). Those of a *bphT1* product (BphT1) and a *bphT2* product (BphT2) are almost identical (97%). BphS, which represents both BphS1 and BphS2, and BphT, which represents both BphT1 and BphT2, are thought to be the HK and RR, respectively. The inducing substrate spectrum of the BphS2T2 system is broad and contains benzene, toluene, ethylbenzene, xylenes, isopropylbenzene, and chlorinated benzenes (9,10). It is the same as that of the BphS1T1 system, except for biphenyl, which is an inducing substrate only for the BphS1T1 system.

In a previous study, the transcriptional start sites for *bphAap*, *etbAa1p*, and *etbAa2p* were determined, and the 18-bp consensus sequence, CcGTAgTTTttcCGGATG (lowercase letters indicate that the consensus is not unanimous), was found 32 to 34 bp upstream from the transcriptional start site for each promoter (8). A similar sequence was also shared with the regions upstream from the *etbD1* and *etbAd* genes, but the distances between the consensus sequence and their transcriptional start sites have yet to be fully elucidated. Deletions in *bphAap* that affected the 18-bp consensus sequence impaired the inducible promoter activity, suggesting the significance of this consensus. The 18-bp consensus sequence appears to be involved in transcriptional induction by the BphS1T1 and BphS2T2 systems.

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doi:10.1016/j.jbiosc.2011.10.021

In this study, the distances between the 18-bp consensus sequence and the transcriptional start sites of the *etbAd* and *etbD1* genes were determined to address the significance of the location of the consensus sequence in the promoters, *etbAdp* and *etbD1p*. The functional significance of the 18-bp consensus sequence was examined using a hybrid promoter between the 18-bp consensus sequence of *bphAap* and the promoter sequence of the benzoate dioxygenase gene in strain RHA1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions The strains and plasmids used in this study are listed in Table 1. *R. jostii* RHA1 was grown at 30°C in 1/5 LB (2 g/l Bacto-tryptone, 1 g/l yeast extract, and 5 g/l NaCl). Host strains, *Rhodococcus erythropolis* IAM1399 (ATCC15963) and *Escherichia coli* JM109 were grown at 30°C and 37°C, respectively, in LB (10 g/l Bacto-tryptone, 5 g/l yeast extract, and 5 g/l NaCl). If necessary, antibiotics were used in the following concentrations: ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (50 μg/ml).

DNA manipulations and analysis DNA manipulations, including electrotransformation (electroporation), were performed as previously described (3,17,18). Nucleotide sequences were determined by the dideoxy termination method using a CEQ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA). A Sanger reaction (19) was performed using the CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter, Inc.). Sequence analysis was performed using BioEdit (20) ApE-A plasmid editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/), and in silico MolecularCloning (in silico biology, Inc., Yokohama, Japan). Sequence logos were generated using WebLogo (http://weblogo.berkeley.edu/) (21,22).

Primer extension analysis The methods of total RNA preparation and determination of transcriptional start site were described previously (23). To determine the transcriptional start site for *etbAdp*, RHA1 was grown at 30°C for 12 h in 10 ml of 1/5 LB and was incubated at 30°C for 16 h in either the presence or absence of ethylbenzene, supplied as vapor. Total RNA from the cells was isolated and subjected to primer extension using the PEXAd primer that had been labeled with Beckman dye D2 (Beckman Coulter, Inc.), which hybridizes to the sequence located between 185 and 209 bp upstream from the initiation codon of *etbAd*. To determine the transcriptional start site for *etbD1p*, *R. erythropolis* IAM1399 harboring pKLAED1 and pFST1 was grown in LB containing kanamycin and chloramphenicol, washed with 1/5 LB, and then suspended in 10 ml of 1/5 LB containing kanamycin and chloramphenicol at an optical density at 600 nm (OD_{600}) of 1.0. pKLAED1 consists of pKLA1 and a 1.2-kb EcoRI-XhoI fragment from RHA1 containing *etbD1p* (16), and pFST1 contains *bphS1T1*. Cell suspension was incubated at 30°C for 16 h in either the absence or presence of ethylbenzene. Total RNA from the cells was isolated and subjected to primer extension using the PEXD1 primer labeled with Beckman dye D4 (Beckman Coulter, Inc.). PEXD1 hybridizes to the sequence located between 102 and 126 bp, downstream from the initiation codon of *etbD1* in pKLAED1. The nucleotide sequences for PEXAd primer and PEXD1 primer are listed in Table 2.

Construction of hybrid promoters and mutations The hybrid promoter fragments in pKLAb2, pKLAbA, and pKLAbA2 were generated by PCR using pT7BA as templates. pT7BA is a derivative of pT7Blue T-Vector containing the *benA* promoter (*benAp*). The primers that were used had SalI sites at their 5′ termini. The nucleotide sequences for the primers are listed in Table 2. Each amplified fragment was subcloned into the pT7Blue T-Vector to verify its nucleotide sequence. The SalI fragments from the resulting plasmids were cloned into the SalI site of the promoter probe vector, pKLA1 (16). The mutant hybrid promoter fragments of pKLAbA2A24, pKLAbA2A23, pKLAbA2A4, pKLAbA2G3, pKLAbA2T2, and pKLAbA2T1 were constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), with pT7bA2 as templates as well as the corresponding primers. The nucleotide sequences for the primers are listed in Table 2. Each of the resulting plasmids was subjected to DNA sequencing to verify the nucleotide sequence of the promoter fragment of interest. The SalI fragments from the resulting plasmids were cloned into the SalI site of the promoter probe vector, pKLA1.

Luciferase assay *R. erythropolis* IAM1399 transformants were grown in LB containing the appropriate antibiotics, washed with 1/5 LB, and suspended in 10 ml of 1/5 LB containing the appropriate antibiotics at an OD_{600} of 1.0. Each cell suspension was incubated at 30°C for 5 h in either the presence or absence of ethylbenzene, which was supplied as vapor. Luciferase activity was measured as previously described (23).

RESULTS

Primer extension analysis of *etbAd* and *etbD1* Primer extension analysis was performed to determine the transcriptional start sites for *etbAdp* and *etbD1p*. For *etbAdp*, total RNA isolated from RHA1 was reverse transcribed using the labeled PEXAd primer, which was complementary to the bases upstream from the initiation codon of *etbAd* (Fig. 1C). Because RHA1 contains an *etbD1*-homolog, *etbD2*, total RNA was isolated from *R. erythropolis* IAM1399 harboring pKLAED1 and pFST1, which contained *etbD1p* and *bphS1T1*, respectively, and was reverse transcribed using the labeled PEXD1 primer, which was complementary to the bases downstream from the initiation codon of *etbD1* (Fig. 1D). Intense peaks for *etbAd* and *etbD1* were observed with only the RNAs isolated from ethylbenzene-induced cells (Figs. 1A and B). The transcriptional start site for *etbAdp* was mapped at 341 nucleotides upstream from the initiation codon of *etbAd*. The 18-bp consensus sequence is located 32 bp upstream from the transcriptional start site for *etbAdp* (Fig. 1C). The major transcriptional start site for *etbD1p*, which is indicated as P1 in Fig. 1B, was mapped to 43 nucleotides upstream from the initiation codon of *etbD1*. The minor one, which is indicated as P2 in Fig. 1B, was mapped to 48 nucleotides upstream from that of *etbD1*. The 18-bp consensus sequence is located 34 bp upstream from the major transcriptional start site for *etbD1p* (Fig. 1D). The *etbAdp* and *etbD1p* sequences were then aligned with the *bphAap*, *etbAa1p*, and *etbAa2p*

TABLE 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Reference or origin
Strains		
R. jostii RHA1	PCB degrader, BPH^+, ETB^+, Nal^r	5
R. erythropolis IAM1399 (=ATCC 15963)	Wild type, BPH^-, ETB^-	IAM culture collection
Escherichia coli JM109	*endA1, glnV44, thi-1, relA1, gyrA96, recA1, mcrB*$^+$, *Δ(lac-proAB)e14-, hsdR17*(r_K^- m_K^+) [F′ *traD36, proAB*$^+$, *lacI*^{q}*ZΔM15*]	13
Plasmids		
pBAB62	pBSL with a 6.2-kb ApaI-BglII fragment containing *bphS1T1*, Ap^r	10
pBluescript II KS(+)	Cloning vector, Ap^r	Stratagene
pBSL	pBluescript II KS(+) with a SpeI site in place of KpnI site, Ap^r	10
pFAJ2574	*Rhodococcus-E. coli* shuttle vector, Cm^r	14
pFST1	pFAJ2574 with a 6.2-kb SpeI fragment containing *bphS1T1* of pBAB62	8
pK4	*Rhodococcus-E. coli* shuttle vector, Km^r	15
pK4BA	pK4 with a 1.9-Kb KpnI-BglII fragment carrying *benA*, Km^r	1
pKLA03	pKLA1 with a 0.2-kb SalI fragment of containing *bphAap*	8
pKLA1	pK4 with a 2.4-kb luciferase gene, *luxAB* from *Vibrio harveyi*, Km^r	16
pKLAED1	pKLA1 with a 1.2-kb EcoRI-XhoI fragment containing *etbD1p*, Km^r	16
pT7Aa03	pT7Blue T-Vector with a 0.2-kb SalI fragment of pKLA03 containing *bphAap*, Ap^r	This study
pT7BA	pT7Blue T-Vector with a 0.6-kb PstI-KpnI fragment of pK4BA containing *benAp*, Ap^r	This study
pT7Blue T-Vector	TA Cloning vector, Ap^r	Novagene
pT7bA2	pT7Blue T-Vector with a 0.1-kb SalI fragment containing hybrid promoter consisting of 24-bp extending consensus sequence and *benAp*, Ap^r	This study

[a] BPH^+, growth on biphenyl; BPH^-, no growth on biphenyl; ETB^+, growth on ethylbenzene; ETB^-, no growth on ethylbenzene; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance.