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NOTE

Identification of Two-Component Regulatory Genes Involved in *o*-Xylene Degradation by *Rhodococcus* sp. Strain DK17

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Putative genes for a two-component signal transduction system (*akbS* and *akbT*) were detected near the alkylbenzene-degrading operon of *Rhodococcus* sp. DK17. Sequence analysis indicates that AkbS possesses potential ATP-binding and histidine autophosphorylation sites in the N- and C-terminal regions, respectively, and that AkbT has a typical response regulator domain. Mutant analysis combined with RT-PCR experiments further shows that AkbS is required to induce the expression of *o*-xylene dioxygenase in DK17.

Key words: *Rhodococcus*, two-component signal transduction proteins, alkylbenzenes

Members of the genus *Rhodococcus* demonstrate a remarkable ability to utilize a wide variety of natural organic and xenobiotic compounds, including aliphatic, aromatic, and alicyclic hydrocarbons (Finnerty, 1992; Warhurst and Fewson, 1994; see also the special issue of Antonie Van Leeuwenhoek, volume 74). To date, several gene clusters involved in the degradation of aromatics have been cloned from *Rhodococcus* spp. These genes are namely degradative genes for biphenyl from *Rhodococcus* sp. strain M5 (Wang *et al.*, 1995), *Rhodococcus globerulus* P6 (Asturias *et al.*, 1995), and *Rhodococcus* sp. strain RHA1 (Sakai *et al.*, 2003), isopropylbenzene-degrading genes from *Rhodococcus erythropolis* BD2 (Kesseler *et al.*, 1996), benzoate dioxygenase genes from *Rhodococcus* sp. strain 19070 (Haddad *et al.*, 2001), and indole oxygenase gene from *Rhodococcus* sp. strain T104 (Choi *et al.*, 2004). However, no in-depth genetic work has been reported regarding the abilities of *Rhodococcus* strains to degrade *o*-xylene.

We previously isolated a new *o*-xylene-degrading *Rhodococcus* strain DK17 and reported that the oxidation of *o*-xylene in *Rhodococcus* sp. DK17 is initiated by a ring-oxidizing oxygenase to form 3,4-dimethylcatechol, and the genes encoding the initial steps in alkylbenzene metabolism are present on a 330-kb megaplasmid pDK2 (Kim *et al.*, 2002). In addition, we showed that the same initial oxygenase genes are implicated in the regiospecific oxidation of *m*- and *p*-xylene to 2,4-dimethylresocinol and 2,5-dimethylhydroquinone, respectively, although DK17 is incapable of growing on these two xylene isomers (Kim *et al.*, 2003). More recently, we completely cloned and sequenced a 37-kb DNA region that contains gene clusters involved in the degradation of alkylbenzenes, including the *o*-xylene dioxygenase genes (Kim *et al.*, 2004; Kim *et al.*, 2005). Two putative transcriptional regulatory genes, designated *akbS* and *akbT*, were discovered near the *o*-xylene operons. Thus, as a part of efforts to understand the *o*-xylene metabolism by DK17 in more detail at the molecular level, the present work was initiated. In this study, we investigated the function of the putative regulatory genes based on gene sequence analysis and mutant characterization.

Since the first characterization of the genes encoding a receptor-like histidine kinase (BpdS) and a cognate response regulator (BpdT) from biphenyl/polychlorinated biphenyl (BP/PCB)-degrading *Rhodococcus* sp. strain M5 (Labbe *et al.*, 1997), several similar genes have been reported in BP/PCB-degrading *Rhodococcus* sp. RHA1 (Takeda *et al.*, 2004), *R. erythropolis* TA421 (GenBank

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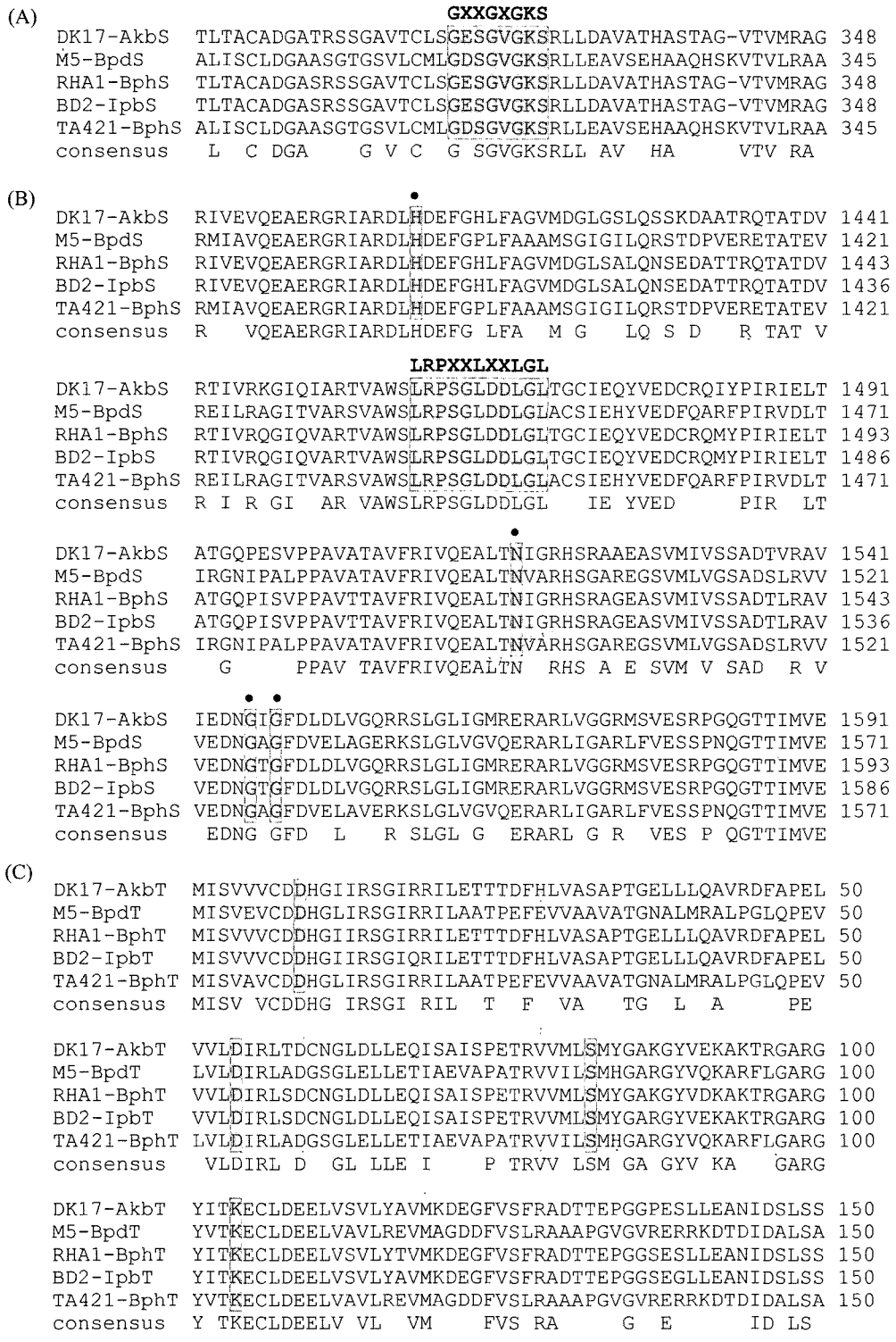

Fig. 1. Conservation of key amino acid residues forming important functional motifs in the two-component signal transduction proteins from various aromatic-degrading *Rhodococcus* spp. Alignment of the N-terminal regions of AkbS and its rhodococcal homologues showing a putative ATP-binding motif (A), presence of conserved sequence blocks at the C-terminal regions of AkbS and its rhodococcal homologues (B), and alignment of AkbT with its rhodococcal homologues (C). The alignment was performed with the BOXSHADE multiple alignment program in Biological software (http://bioweb.pasteur.fr). Important and conserved amino acid residues are shaded gray. DK17-AkbS and DK17-AkbT, a histidine kinase and a response regulator from *Rhodococcus* sp. strain DK17; M5-BpdS and M5-BpdT, a histidine kinase and a response regulator from *Rhodococcus* sp. strain M5 (Labbe *et al.*, 1997); RHA1-BphS and RHA1-BphT, a histidine kinase and a response regulator from *Rhodococcus* sp. strain RHA1 (Takeda *et al.*, 2004); BD2-IpbS and BD2-IpbT, a histidine kinase and a response regulator from *R. erythropolis* BD2 (Stecker *et al.*, 2003); TA421-BphS and TA421-BphT, a histidine kinase and a response regulator from *R. erythropolis* TA421 (GenBank accession No. AB014348).

accession No. AB014348), and isopropylbenzene-degrading *R. erythropolis* BD2 (Stecker *et al.*, 2003). BLASTP analysis showed that DK17-AkbS (1,596 amino acid residues) is well-matched with the sensor kinases from the aromatic-degrading *Rhodococcus* spp. listed above (91, 89, 54, and 54% identity to RHA1-BphS, BD2-IpbS, M5-BpdS, and TA421-BphS, respectively, over at least 98% of the DK17-AkbS total length with zero E values). Labbe *et al.* (1997) previously reported that the predicted histidine kinase (BpdS) is an unusually large transmembrane protein which contains ATP-binding, leucine-rich repeat motifs, as well as a number of conserved residues of protein kinases. The alignments shown in Fig. 1 extend this observation to all of the currently known receptor-like histidine kinases from aromatic-degrading *Rhodococcus* spp. As shown in Fig. 1A, an ATP-binding motif, GXXGXGKS (Walker *et al.*, 1982), was located at the amino terminus of the alignment, which is perfectly conserved in all of the aligned sequences. A carboxy-terminal alignment reveals the presence of conserved sequence blocks known as H, N, G1, and G2 (marked closed dots in Fig. 1B) in the Vsr/ComP/DegS subfamily of histidine kinases (Parkinson and Kofold, 1992; Stock *et al.*, 2000). The invariant histidine is the potential site of histidine autophosphorylation, while G1 and G2 are believed to be involved in nucleotide binding (Stock *et al.*, 2000). Additionally, observation of the wholly conserved LRPXXLXXLGL sequence between the H and the N blocks supports the previous speculation that this sequence may represent a submotif of these kinases because it is the most extensive stretch of conserved sequence within the kinase domains (Labbe *et al.* 1997).

Akin to AkbS, AkbT (209 amino acid residues) is also mostly homologous to response regulators from aromatic-degrading *Rhodococcus* spp. (62 - 96% identity). AkbT was found to have a conventional response regulator domain, which is indicated by the presence of conserved Asp^{9} (D), Asp^{54} (D), Ser^{82} (S), and Lys^{104} (K) (Fig. 1C) (Volz, 1993). It should be noted that the termination codon (TGA) of *akbS* overlaps the ATG start codon of *akbT*. This observation suggests that there is a translational coupling between *akbS* and *akbT*, which may, in turn, ensures the stoichiometric synthesis of the AkbS and AkbT proteins. Indeed, Larkin *et al.* (1998) proposed that such a control mechanism makes sense for gene products, which are incorporated into large assemblies in a 1:1 ratio.

By treatment with UV light, we previously generated a total of 54 mutant strains incapable of growing on *o*-xylene. We subsequently found that 49 mutant strains neither consumed *o*-xylene nor oxidized indole to indigo when grown on glucose in the presence of *o*-xylene vapors. This indicates the loss of initial oxygenase activity. Due to the loss of a 330-kb megaplasmid pDK2, the mutant strain, DK176, from this group is unable to grow on *o*-xylene, ethylbenzene, isopropylbenzene, toluene, and benzene (Kim *et al.*, 2002). In order to determine whether all of the mutant strains in this group had lost pDK2, pulsed-field gel electrophoresis (PFGE) analysis of genomic DNA was performed to separate large plasmids from one another and from the chromosome, as previously described (Kim *et al.*, 2002). Interestingly, as shown in Fig. 2, one mutant strain designated as DK183 was found to retain pDK2, although it shows the same growth phenotype as DK176, the pDK2-cured strain (Table 1). Since indigo production from indole is characteristic of the initial *o*-xylene dioxygenase (Kim *et*

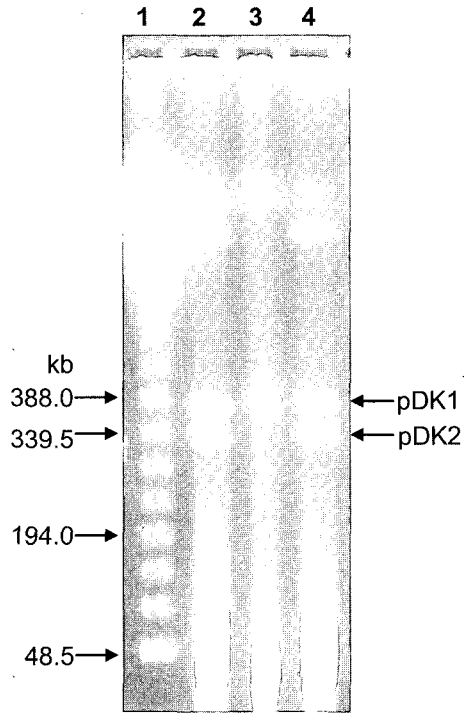

Fig. 2. PFGE separation of genomic DNAs prepared from *Rhodococcus* sp. strains DK17, DK176, and DK183. Lane 1, λ ladder standard; lane 2, *Rhodococcus* sp. strain DK17; lane 3, *Rhodococcus* sp. strain DK176; lane 4, *Rhodococcus* sp. strain DK183. PFGE was performed using a Bio-Rad Laboratories CHEF DRIII system. Gels [1.0% agarose in 0.5 x TBE buffer (1x TBE is 89 mM Tris borate, 2.5 mM EDTA, pH 8.0)] were run at 6 V/cm at 14°C. The pulse duration increased from 15 to 60 sec during a 16 h run.

Table 1. Growth characteristics of *Rhodococcus* sp. strains DK17, DK176, and DK183 on various monocyclic aromatic hydrocarbons

Compound	DK17	DK176	DK183
o-Xylene	++++ [a]	- [b]	-
Ethylbenzene	++++	-	-
Isopropylbenzene	++++	-	-
Toluene	++	-	-
Benzene	+	-	-
Phenol	+++	+++	+++

[a]Four, three, two, and one plus signs indicate the formation of 1.0 mm-in-diameter colony within 3, 5, 7, and 9 days, respectively.
[b]A minus sign denotes that the compound could not serve as the sole carbon and energy source.

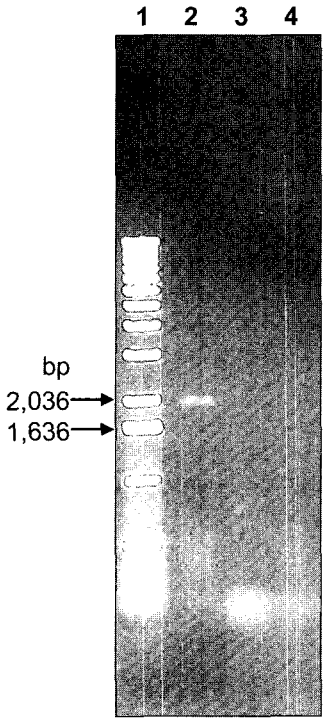

Fig. 3. Agarose gel electrophoresis of RT-PCR products from total RNA. Lane 1, molecular weight markers; lane 2, DK17 grown in the presence of *o*-xylene; lane 3, DK183 grown in the presence of *o*-xylene; lane 4, DK17 grown only on glucose.

al., 2002; Kim *et al.*, 2004), DK183 is believed to bear a mutation in either *o*-xylene dioxygenase genes or in the gene which encodes the regulatory or transport protein. In order to verify this hypothesis, *o*-xylene dioxygenase genes were amplified by PCR and sequenced completely. Alignment of DK183-derived dioxygenase sequences with those of DK17 revealed that the *o*-xylene dioxygenase genes from DK183 possess the same nucleotide sequences as those of the wild-type strain, DK17 (data not shown).

The above results suggest that DK183 mutant has a mutation in a gene which encodes regulatory or transport functions. Accordingly, using the nucleotide sequence as a guide, we further determined that the mutant strain, DK183, has a nonsense mutation in the 1,350th codon of the *akbS* gene while the sequence of the *akbT* gene is intact. The GAG codon for glutamate had been changed to a UAG stop codon, truncating the gene to less than 85% of its normal size of 1,596 amino acid residues. As explained previously, the potential histidine kinase domains, such as the histidine autophosphorylation site and kinase submotif, are located near the C-terminal region of AkbS (residues 1,409 - 1,548). Thus, the mutation in DK183 is predicted to result in the production of a truncated AkbS protein (1,349 amino acid residues) without the putative histidine kinase motifs. To further corroborate this postulation, RT-PCR experiments were carried out with total RNAs extracted from *o*-xylene-induced cells of DK17 and DK183.

Rhodococcus sp. strains DK 17 and DK183 cells reaching the exponential phase in 50 ml of mineral salts basal (MSB) medium (Stanier *et al.*, 1966) containing 20 mM glucose were separately harvested and resuspended in 50 ml of the same fresh medium. In order to induce the *o*-xylene dioxygenase genes, *o*-xylene was added directly to each suspension at a final concentration of 0.1% (v/v) and further incubated at 30°C for 12 h. A negative control was prepared without provision of *o*-xylene. Total RNA extraction was performed as described by Mahenthiralingam (1998). The extracted total RNA was further purified by spin column and DNase I treatment according to the manufacturer's instructions (QIAGEN, Germany). It was then subjected to PCR with and without a prior reverse transcription (RT) step with primers, which were designed based on the sequence of the gene encoding an *o*-xylene dioxygenase oxygenase component (*akbA1A2*) (forward primer, 5'-ATGGAGTGGAGCATGTTGA-3'; reverse primer, 5'-TCAGAGGAAGATGTTGAG-3'). RT-PCR reactions were performed in 25 μl reaction mixtures containing 70 ng of total RNA and 25 pmol of each primer with QIAGEN OneStep RT-PCR Enzyme Mix (QIAGEN, Germany). The thermocycler program used for the RT-PCR reactions was as follows: 50°C for 30 min, 95°C for 15 min, 30 X (94°C for 30 s, 55°C for 30 s, 72°C for 1 min), and 72°C for 10 min. As shown in Fig. 3, the expected size of the RT-PCR product (1,941 bp) was amplified only from the wild-type cells induced by *o*-xylene, while the no RT-PCR product was detectable in the *o*-xylene-induced cells of DK183, as well as the negative control. The results of this mutant analysis indicate that the protein encoded by *akbS* is required to induce the expression of *o*-xylene dioxygenase in DK17. Overall, combined with the sequence homology data, the present work strongly suggests that the *akbS* and *akbT* genes together encode two-component signal transduction proteins which play a crucial role in regulating the metabolism of alkylbenzenes, including *o*-xylene in DK17.

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