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Transcriptional Activation of the *bkd* Operon of *Pseudomonas putida* by BkdR

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Reinvestigation of the transcriptional start site of the *bkd* operon of *Pseudomonas putida* revealed that the transcriptional start site was located 86 nucleotides upstream of the translational start. There was a σ^{70} binding site 10 bp upstream of the transcriptional start site. The dissociation constants for BkdR, the transcriptional activator of the *bkd* operon, were 3.1×10^{-7} M in the absence of L-valine and 8.9×10^{-8} M in the presence of L-valine. Binding of BkdR to substrate DNA in the absence of L-valine imposed a bend angle of 92° in the DNA. In the presence of L-valine, the angle was 76°. BkdR did not bind to either of the two fragments of substrate DNA resulting from digestion with *Age*I. Because *Age*I attacks between three potential BkdR binding sites, this suggests that binding of BkdR is cooperative. *P. putida* JS110 and JS112, mutant strains which do not express any of the components of branched-chain keto acid dehydrogenase, were found to contain missense mutations in *bkdR* resulting in R40Q and T22I changes in the putative helix-turn-helix of BkdR. Addition of glucose to the medium repressed expression of *lacZ* from a chromosomal *bkdR-lacZ* fusion, suggesting that catabolite repression of the *bkd* operon was the result of reduced expression of *bkdR*. These data are used to present a model for the role of BkdR in transcriptional control of the *bkd* operon.

The branched-chain keto acid dehydrogenase complex of *Pseudomonas putida* is the second enzyme in the pathway for the metabolism of branched-chain amino acids. There are three components in the complex, E1, the dehydrogenase, E2, the transacylase, and E3, the lipoamide dehydrogenase. The E1 component of *P. putida* branched-chain keto acid dehydrogenase has been purified and shown to be a heterotetramer (10). The E3 component is a specific lipoamide dehydrogenase, LPD-val (27), which has been crystallized and whose structure has been solved (19). The four polypeptides constituting branched-chain keto acid dehydrogenase are encoded by the *bkd* operon (2–4), which is expressed as a polycistronic message.

Branched-chain keto acid dehydrogenase of *P. putida* is induced during growth on branched-chain amino or keto acids (18). Expression is also under catabolite repression control by glucose and succinate (28). Expression of the *bkd* operon is positively regulated by BkdR, which is encoded by a structural gene which is divergently transcribed from the *bkd* operon (17). BkdR is a homolog of Lrp, the leucine-responsive protein of *Escherichia coli* (5). Lrp is a global regulator in *E. coli*, but the low copy number of BkdR suggests that its main purpose in *P. putida* is regulation of the *bkd* operon (16). The region to which BkdR binds was identified by DNase I protection studies (16) as a large segment of DNA between *bkdR* and *bkdA1*, the latter being the first gene of the *bkd* operon (Fig. 1). BkdR is a homotetramer (14, 16), and three tetramers of BkdR bind to its substrate DNA (12). Millimolar L-branched-chain amino acids cause a conformational change in BkdR (14), and the fact that L-branched-chain amino acids also affect the DNase I protection pattern suggested that L-branched-chain amino acids are the inducers of the pathway rather than branched-chain keto acids.

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This report presents additional information about the role of BkdR in transcription of the *bkd* operon, including the transcriptional start site, DNA bending, and catabolite repression. A model is presented for the transcriptional activation of the *bkd* operon by BkdR.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are shown in Table 1. Valine-isoleucine medium (18) contained 0.3% valine and 0.1% isoleucine and was used for the growth of *P. putida* PpG2, since growth with both amino acids is faster than with valine alone. The data in Table 3 were obtained with cells of *P. putida* JS386 grown in valine-isoleucine medium but with these amino acids as the sole source of nitrogen. Although *P. putida* JS386 is unable to make branched-chain keto acid dehydrogenase, it can grow slowly on this medium because it is able to transaminate valine and isoleucine. When 10 mM L-glutamate was the sole carbon source, the same basal salt mixture was used. *P. putida* was grown with aeration at 30°C, and *E. coli* was grown with aeration at 37°C. The final concentrations of antibiotics necessary to inhibit the growth of *P. putida* were 90 μg/ml for kanamycin and 200 μg/ml for tetracycline. The concentrations of ampicillin and kanamycin necessary to inhibit the growth of *E. coli* were 200 and 90 μg/ml, respectively.

Enzymes and chemicals. Restriction endonucleases and other DNA-modifying enzymes were obtained from Promega (Madison, Wis.) and GIBCO Bethesda Research Laboratories (BRL) (Gaithersburg, Md.). The double-stranded (ds) DNA Cycle Sequencing System and SuperScript II RNase H^- reverse transcriptase were from GIBCO BRL, and the *fmol* DNA Cycle Sequencing System was obtained from Promega. [α-^{32}P]dCTP and [γ-^{32}P]ATP were from Dupont, NEN Research Products (Boston, Mass.); *Pseudomonas* isolation agar was from Difco Laboratories (Detroit, Mich.). The Sequenase 7-Deaza-dGTP DNA Sequencing Kit was from United States Biochemical (Cleveland, Ohio). pBend2 (13) was obtained from S. Adhya (National Cancer Institute, Bethesda, Md.). Synthetic oligonucleotides were prepared by the Molecular Biology Resource Facility, William K. Warren Medical Research Institute, University of Oklahoma Health Sciences Center, Oklahoma City.

Nucleic acid isolation and analysis. Chromosomal and plasmid DNAs were purified by cesium chloride gradients. Small-scale isolation of plasmids by alkaline lysis, restriction digestions, blunt ending of restriction fragments, DNA labeling, and ligations were done as described by Sambrook et al. (25). Total cellular RNA and mRNA were prepared as described previously (15). Gel mobility shift assays were conducted as described earlier (16), with a final concentration of 50 mM L-valine when it was used.

Enzymic methods. Preparation of *P. putida* PpG2 cell extracts containing pKRZ-1-derived plasmids and the measurement of β-galactosidase activity were

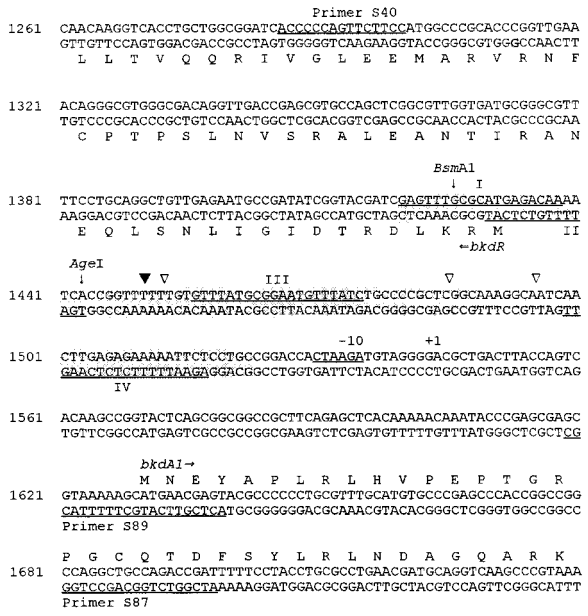

FIG. 1. Nucleotide sequence of the *bkdR-bkdA1* intergenic region. The numbering is the same as that used in GenBank sequence M57613. The bases protected from the action of DNase I by BkdR are shaded (16). The transcriptional start site for the *bkd* operon is designated +1. The nucleotides corresponding to the −10 position are underlined. The bend center is marked with the symbol ▼, and hypersensitive sites are marked with the symbol ▽ (16). I, II, III, and IV are potential BkdR-binding sites based on similarity to Lrp-binding sites (7, 16, 31). *Age*I and *Bsm*AI restriction sites are marked. The primers used are underlined and identified by designation.

done as described in reference 20. Isolation of BkdR from *E. coli* DH5α (pJRS119) was done as described in reference 16.

Reverse transcriptase mapping. Ten to 25 μg of total cellular RNA extracted from *P. putida* was mixed with 5 pmol of 5′-end-labeled oligonucleotide S87 or S89 (Fig. 1). The sequence of S87, 5′-ATC GGT CTG GCA GCC TGG-3′, is identical to nucleotides (nt) 1698 to 1681 on the bottom strand of Fig. 1. The sequence of S89 is 5′-ACT CGT CCA TGG TTT TTA CGC-3′. S89 is a mutagenizing primer designed to introduce an *Nco*I site into *bkdA1* and matches the bottom strand in Fig. 1 between nt 1639 and 1619 of GenBank sequence M57613. The RNA-primer mixture was denatured, and the primer was extended by using SuperScript II RNase H$^-$ reverse transcriptase for 30 min at 40°C in accordance with the manufacturer's recommendations. The cDNA was precipitated with ethanol and electrophoresed on a DNA sequencing gel to analyze the products of primer extension (25). Dideoxy sequencing reactions were performed by using S87 and pJRS25 as the template (15). The reaction products were electrophoresed alongside the primer extension products to locate the position of the +1 nucleotide.

Plasmid constructions. pJRS136 contains a complete copy of *bkdR* under the control of the *tac* promoter in the broad-host-range cloning vector pVLT33 (8). The first step was digestion of pJRS40 (15) with *Xba*I, which cuts in the pUC19 polylinker, and then blunt ending it with Klenow reagent and α-phosphorothioates, which rendered this end of the DNA fragment insensitive to exonuclease attack. The plasmid was then digested with *Not*I, which released a 293-bp fragment from the *bkdR-bkdA1* intergenic region. Deletions were created from the *Not*I end by digesting with 25 U of exonuclease at 37°C with the Erase-a-Base kit (Promega), and samples were withdrawn at 30 and 60 s. The digest was then ligated, and the extent of the deletion of the *bkdR* promoter region was determined by ds sequencing with the pUC forward primer (25). One clone having an insert of nt 1 to 1447 of GenBank DNA sequence M576713 was selected for cloning into pVLT33 (8). This region includes a partial open reading frame with sequence similarity to *glnA*, all of *bkdR*, and 16 nt upstream of the start codon of *bkdR*. This fragment was transcloned from pUC19 to pVLT33 by using the *Eco*RI and *Hin*dIII sites of both vectors. The resulting plasmid, pJRS136, was used to transform *P. putida* JS110 and JS112, which do not express any of the components of branched-chain keto acid dehydrogenase and therefore are unable to grow on valine-isoleucine agar. *P. putida* JS110 and JS112 harboring pJRS136 were able to grow on valine-isoleucine agar only if isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium. These same strains harboring pVLT33 were unable to grow on these media, even with IPTG.

The location of the transcriptional start site of the *bkd* operon was confirmed by the use of ordered *lacZ* transcriptional fusions flanking the +1 nucleotide (Fig. 2). DNA fragments were synthesized by PCR using a forward primer which was the same for all four fragments. The forward primer matched nt 916 to 935 on the top strand of GenBank sequence M57613. The reverse primers had a *Bam*HI site and were different for each fragment (Fig. 3). The PCR-amplified fragment was cloned into the *Eco*RV site of the pBluescript II SK$^+$ phagemid and used to transform *E. coli* DH5α. Recombinant plasmid clones having *bkdR* in the same orientation as *lacZ* were selected for further cloning. The inserts were sequenced to ensure that there were no unwanted mutations. The insert was released by digesting the pBluescript II-derived plasmids with *Bam*HI and *Sal*I and cloned into the same sites of pKRZ-1 (24) to yield plasmids pJRS160 to pJRS163 (Fig. 3). pKRZ-1 is a promoter probe vector with a *lacZ* reporter gene. The recombinant pKRZ-1 constructions were transferred into *P. putida* PpG2 by triparental mating (26).

Dissociation constant of BkdR. The dissociation constant of BkdR for substrate DNA in the presence or absence of L-valine was determined by gel retardation assay. Various amounts of BkdR were added to fragment A (12), which includes bases 1299 to 1678 (Fig. 1). The reaction mixture was incubated at room temperature for 5 min and then subjected to a gel mobility shift assay. The concentration of BkdR that caused half of the substrate DNA to become complexed under the experimental conditions used was taken as the dissociation constant (K_d) of BkdR-DNA complexes.

DNA bending assays. A PCR-amplified fragment was synthesized by using a forward primer (5′-AGG CTC TAG AGA ATG CCG AT-3′) complementary to nt 1388 to 1407 and a reverse primer (5′-CAT CTT AGT CGA CCG GCA GG-3′) complementary to nt 1537 to 1518 of GenBank sequence M57613. The forward and reverse primers have *Xba*I and *Sal*I sites, respectively. The amplified product was digested with *Xba*I and *Sal*I, and the 137-bp product (bp 1393 to 1529 in Fig. 1) was cloned into pBend2 (13). The resulting plasmid, pJRS165, was digested with *Mlu*I, *Bgl*II, *Xho*I, *Pvu*I, *Sma*I, *Stu*I, *Nru*I, and *Bam*HI to generate a set of equal-length fragments with different 5′ and 3′ ends. These fragments were labeled at the 3′ end by using Klenow reagent and [α-^{32}P]dCTP. The radiolabeled DNA fragments were incubated with BkdR in the presence or absence of L-valine and analyzed by the gel mobility shift assay at room temperature (16). There was no intrinsic bending of the labeled fragments when they were electrophoresed in the absence of BkdR.

The angle of the bend was estimated by use of the expression $\mu_M/\mu_E = \cos$

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype or description	Source or reference
P. putida strains		
PpG2	Wild type	I. C. Gunsalus
JS110	*bkdR110*	29
JS112	*bkdR112*	29
JS326	Does not express *bkd* operon	29
JS382	Δ*bkdR*	16
JS386	*bkdR-lacZ* fusion	16
E. coli DH5α	*supE44 hsdR17 deoR recA13 endA1 lacZΔM15*	BRL
Plasmids		
pBend2	DNA-bending vector	13
pBluescript II SK$^+$	Phagemid cloning vector	Stratagene
pCYTEXP1	Expression vector	1
pKRZ-1	Promoter probe vector	24
pJRS25	*bkdR*, intergenic region, and part of *bkdA1*	15
pJRS119	BkdR expression vector	16
pJRS136	Promoterless *bkdR* in pVLT33	This study
pJRS160	nt 916 to 1581[a] inserted into pKRZ-1	This study
pJRS161	nt 916 to 1561[a] inserted into pKRZ-1	This study
pJRS162	nt 916 to 1541[a] inserted into pKRZ-1	This study
pJRS163	nt 916 to 1528[a] inserted into pKRZ-1	This study
pJRS165	135-bp PCR product cloned into pBend2 vector	This study
pRK2013	Helper plasmid	9
pVLT33	Cloning vector	8

[a] Nucleotides are numbered in accordance with GenBank sequence M57613.

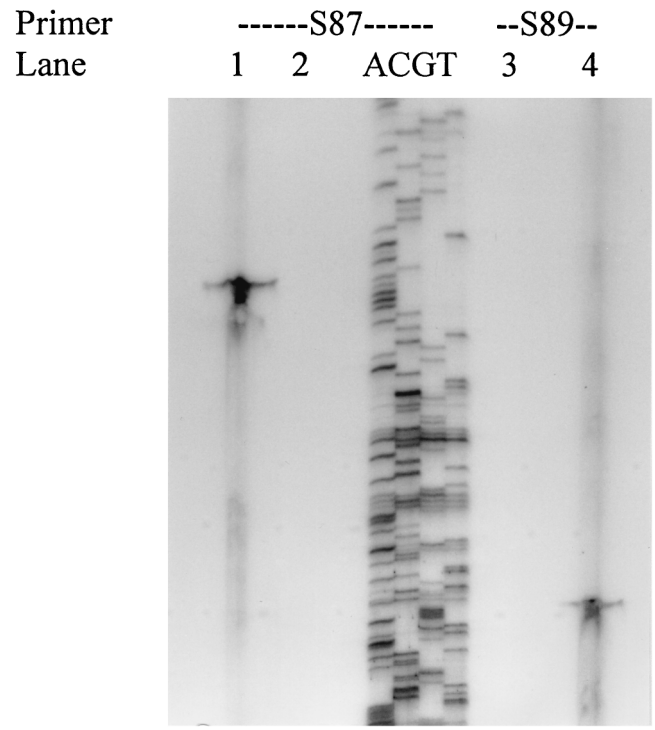

FIG. 2. Primer extension analysis of the *bkd* operon. Total RNA was isolated from *P. putida* PpG2 and JS382 grown in noninducing (glutamate mineral salts) and inducing (valine-isoleucine-glutamate) media. RNA was hybridized to 5′-end-labeled primers S87 and S89 (Fig. 1) and extended by using reverse transcriptase, and the products were analyzed on a sequencing gel. Lanes A, C, G, and T contained the products of a sequencing reaction using primer S87. Lanes 1 and 4 contained primer extension products isolated from *P. putida* grown in glutamate-valine-isoleucine (inducing) medium, and lanes 2 and 3 contained the products of *P. putida* PpG2 grown in glutamate (noninducing) medium. No product was obtained with *P. putida* JS382, which has a deletion in *bkdR*.

($\alpha/2$), where μ_M and μ_E represent the gel mobilities of molecules with bends at their centers and ends, respectively, and α is the angle by which the DNA departs from linearity (13).

Additional information about binding of BkdR around the bend center of substrate DNA was obtained by a study of the ability of BkdR to effect a gel shift of DNA fragments resulting from the digestion of substrate DNA. A 354-bp DNA fragment containing nt 1286 to 1639 (Fig. 1) was synthesized by PCR amplification and 3′ end labeled by using Klenow reagent, [α-^{32}P]dCTP, and deoxynucleoside triphosphates. The primers used to create this DNA fragment were S40 (Fig. 1) and S89. S89 was described in the paragraph in this section on reverse transcriptase mapping. The composition of S40 is 5′-ACC CCC AGT TCT TCC-3′, which is identical to the top strand of Fig. 1 from nt 1286 to 1300. The 354-bp fragment was digested with *Age*I and *Bsm*AI, and the 3′ ends were filled in with [α-^{32}P]dCTP, deoxynucleoside triphosphates, and Klenow reagent. After filling in, the lengths of the two *Age*I fragments were 161 and 196 bp, and those of the two *Bsm*AI fragments were 145 and 205 bp. The DNA-binding ability of BkdR was tested with the end-labeled restriction fragments by the gel mobility shift assay in the presence and absence of L-valine.

Identification of mutations affecting *bkdR*. To identify the location of *bkd* mutations of *P. putida* JS110, JS112, and JS326, *bkdR* was isolated from each of these mutants by PCR amplification employing a forward primer matching nt 916 to 935 and S89 as the reverse primer. The PCR product includes all of *bkdR*, the intergenic region, and 10 nt of *bkdA1*. Five micrograms of purified chromosome of *P. putida* PpG2 was used as the template. The nucleotide sequence of the 783-bp amplified product was determined by using the ds DNA Cycle Sequencing System (GIBCO BRL). The mutations were confirmed by three independent amplifications of *bkdR* from the mutant chromosome followed by nucleotide sequencing.

RESULTS

Location of the transcriptional start of the *bkd* operon. An earlier report located the transcriptional start of the *bkd* operon at what turned out to be inside *bkdR* (15), which seemed unlikely. Therefore, the transcriptional start site of the *bkd* operon was reinvestigated by primer extension from two separate primers. Two strains of *P. putida* were used for this study, PpG2, which is the wild type, and JS382, which has a deletion in *bkdR* (17) and was used as a negative control. Both strains were grown in synthetic media with glutamate as the noninducing carbon source and with glutamate-valine-isoleucine as the inducing medium. Since *P. putida* JS382 is unable to grow in valine-isoleucine medium, glutamate was added as a metabolizable carbon source. Total cellular RNA was extracted from the cells and extended from primers S87 and S89 by using reverse transcriptase as described in Materials and Methods. The product obtained by extending S87 was 155 nt long, and the product obtained by extending S89 was 96 nt long (Fig. 2). Both products terminated at nt 1544, which is an adenine (Fig. 1). No product was obtained with RNA from PpG2 grown on glutamate-mineral salts medium (Fig. 2) or with RNA isolated from *P. putida* JS382 grown in the valine-isoleucine-glutamate medium (data not shown).

The location of the transcriptional start site was confirmed by using a set of ordered transcriptional *lacZ* fusions flanking the presumed *bkd* promoter (Fig. 3). The DNA fragments shown in Fig. 3 were created by PCR (see Materials and Methods) and cloned into pKRZ-1, a broad-host-range promoter probe vector with a *lacZ* reporter gene (24). The DNA fragments extended from nt 916 to nt 1581 (pJRS160), 1561 (pJRS161), 1541 (pJRS162), and 1528 (pJRS163) and included *bkdR*. These plasmids were then used to transform *P. putida* PpG2. The data in Table 2 show that fragments which terminated on the 3′ side of the +1 site (pJRS160 and pJRS161) expressed high levels of β-galactosidase. The fragment which terminated at −3 relative to the +1 site (pJRS162 in Table 2) had only 10% of the activity of pJRS160 and pJRS161. The fragment which terminated at −16 relative to the transcriptional start site (pJRS163) had the same activity as the control, pKRZ-1.

pJRS163 completely lacks the putative RNA polymerase binding site, and pJRS162 lacks most of it. These results are consistent with a transcriptional start site at nt 1544. There is

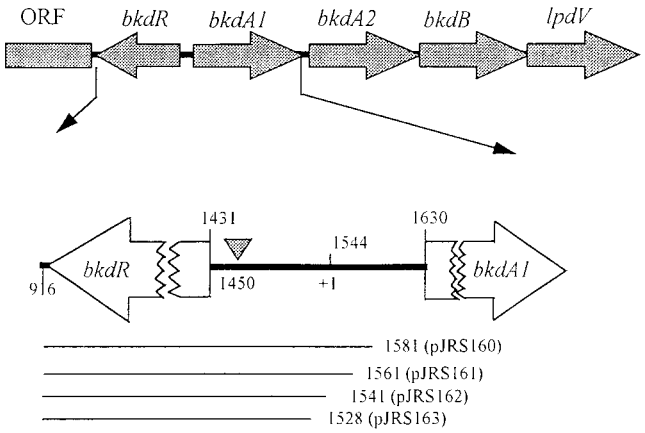

FIG. 3. The *bkd* operon and ordered *lacZ* transcriptional fusions used to identify the *bkd* promoter and transcriptional start site. The numbers are the 3′ end of the promoter probe clones and correspond to the numbering of GenBank nucleotide sequence M57613. The genes of the *bkd* operon are *bkdA1* and *bkdA2*, which encode the α and β subunits of the E1 component, *bkdB*, which encodes E2 (the transacylase), and *lpdV*, which encodes LPD-val, the E3 component. ORF encodes a protein with sequence similarity to glutamine synthetase, and *bkdR* encodes BkdR. The filled triangle indicates the bend center.

TABLE 2. Effect of progressive deletions in the *bkd* promoter region on expression of *bkdR-lacZ* fusions

Plasmid	3′-end nucleotide no.	β-Galactosidase sp act[a]	SD
pJRS160	+38	3,197	272
pJRS161	+18	3,103	377
pJRS162	−3	306	66
pJRS163	−16	6.8	1.3
pKRZ-1	None	7	1

[a] Nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. These data are averages of three separate experiments.

a consensus −10 site for a σ^{70} promoter (designated −10 in Fig. 1) but no identifiable −35 site, which is usually the case with transcriptional activators (21).

Dissociation constant for binding of BkdR to DNA. To determine the dissociation constant, it is necessary to know if an equilibrium is reached between free DNA and bound DNA and whether the bound complex dissociates during electrophoresis. The BkdR-DNA complexes were incubated for 2 to 30 min at room temperature and then analyzed by the gel mobility shift assay. No differences in the complexes were found, suggesting that the DNA-BkdR complex formation was rapid and essentially irreversible. To answer the second question, a limiting amount of radiolabeled DNA was mixed with BkdR and an excess of unlabeled DNA and analyzed by the gel mobility shift assay for 90 min. Labeled DNA was not replaced by unlabeled DNA, indicating that the complex did not dissociate during electrophoresis.

It was previously shown that BkdR purified by the procedure used in this study was essentially 100% active (12) so that no correction of the K_d was needed for purity. The concentration of BkdR that caused half of the DNA to become complexed under the experimental conditions used was taken as the dissociation constant (K_d) of the BkdR-DNA complex. The K_d values were 3.1×10^{-7} M in the absence of L-valine and 8.9×10^{-8} M in the presence of L-valine.

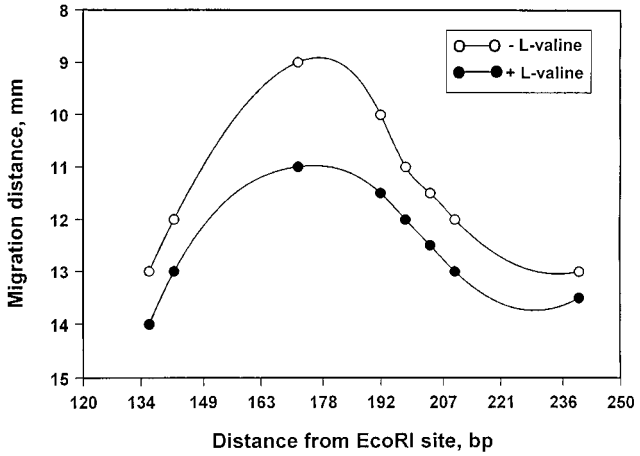

FIG. 4. Migration of circularly permuted DNA fragments containing the *bkdR-bkdA1* intergenic region. The plasmid containing the *bkdR-bkdA1* intergenic region was digested with *Mlu*I, *Bgl*II, *Xho*I, *Pvu*II, *Sma*I, *Stu*I, *Nru*I, and *Bam*HI. The resulting fragments were end labeled with ^{32}P, incubated with BkdR in the presence (■) and absence (●) of 50 mM L-valine, and then analyzed in the gel mobility shift assay. Migration (*y* axis) was plotted as a function of the number of nucleotides to the *Eco*RI site (*x* axis). Each point represents the migration distance of a fragment.

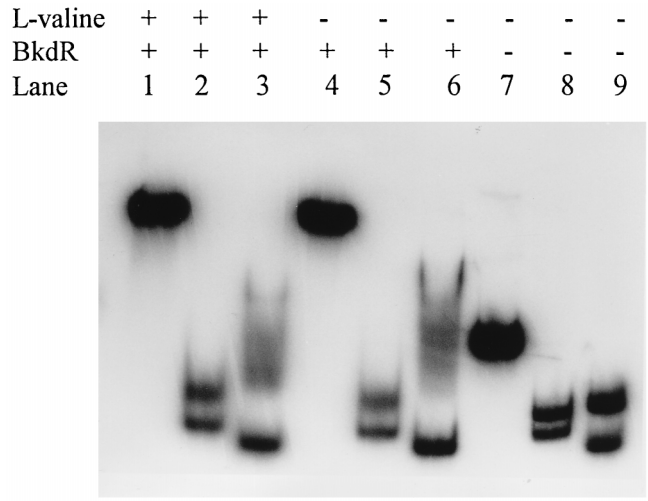

FIG. 5. Effect of digestion of substrate DNA by *Age*I and *Bsm*AI on the binding of BkdR. The 354-bp PCR fragment was digested with *Age*I and *Bsm*AI. The full-length and cut fragments were end labeled and analyzed by the gel mobility shift assay under the incubation conditions described in Materials and Methods. Lanes 1, 4, and 7 contained the full-length fragment, lanes 2, 5, and 8 contained the fragments resulting from digestion with *Age*I, and lanes 3, 6, and 9 contained the fragments resulting from digestion with *Bsm*AI. The concentration of L-valine was 50 mM.

Bending of DNA by BkdR. Transcriptional activators usually bend DNA (21), and the possibility of BkdR-induced bending was investigated by using pBend2 (13), which was designed specifically for this purpose. pBend2 contains a set of restriction sites in tandem into which the DNA fragment is cloned. A 137-bp fragment containing the putative BkdR-binding sites was prepared and cloned into pBend2 by using the *Xba*I and *Sal*I sites. The plasmid construction was digested with *Mlu*I, *Bgl*II, *Xho*I, *Pvu*II, *Sma*I, *Stu*I, *Nru*I, and *Bam*HI, which give fragments of the same length, but with the 137 bp insert at different positions relative to the end of the fragment. In the absence of BkdR, all of the fragments had the same mobility in gel shift assays, but when BkdR was incubated with each fragment, the mobilities varied (Fig. 4). The closer the bend center is to the center of the fragment, the greater the retardation in the gel shift assays, which makes it possible to locate the bend center. The bend center was located at approximately bp 1450 (Fig. 1), in the middle of a string of six T's. The bend angle calculated from the gel shift assays with only BkdR and substrate DNA was 92°. The bend angle calculated from gel shift assays done with 50 mM L-valine was 76°; i.e., the bend angle was smaller with the effector.

Restriction digestion analysis of BkdR-binding sites. Previous studies identified four potential BkdR-binding sites based on similarity to Lrp-binding sites (5). There are two convenient restriction sites in the protected region which were used to study BkdR binding. The site for *Age*I is located between nt 1443 (Fig. 1), which is located close to sites I to III, and the bend center and hypersensitive sites which are enhanced in the presence of L-valine (5). To localize the binding sites more exactly, a 354-bp DNA fragment (nt 1286 to 1639 in Fig. 1) containing the *bkdR-bkdA1* intergenic region was digested with *Age*I and blunt ended, which produced 161- and 196-bp fragments. BkdR bound to the undigested 354-bp fragment but not to either of the restriction fragments (Fig. 5). This strongly suggests that cooperative binding exists between BkdR and the two *Age*I fragments. Addition of L-valine to the BkdR-DNA complex had no effect on binding to the fragments.

TABLE 3. Expression of β-galactosidase from a *bkdR-lacZ* fusion in *P. putida* JS386

Medium	β-Galactosidase sp act[a]	SD
Valine-isoleucine + lactate	293	24.6
Valine-isoleucine + glucose	177	35.8
Histidine + lactate	188	14.6
Histidine + glucose	170	17.7

[a] Nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. These data are averages of three separate experiments.

The site for *Bsm*AI is at nt 1426 (Fig. 1), and digestion of the 354-bp fragment produced 145- and 205-bp fragments. When BkdR was added, the 205-bp fragment shifted but the 140-bp fragment did not (Fig. 5). The *Bsm*AI restriction site is barely within the region protected by DNase I and is within a potential BkdR-binding site (Fig. 1). Again, there was no obvious effect of L-valine on the binding of BkdR.

Carbon regulation of the *bkd* operon. Regulation by carbon can occur either by interference with the function of BkdR and/or RNA polymerase or by interference with the expression of *bkdR*. *P. putida* JS386, which carries a *lacZ* fusion in the *Nco*I site of *bkdR* (16), was used to distinguish between these two possibilities. Since *P. putida* JS386 cannot metabolize branched-chain amino acids, lactate, a nonrepressing carbon source, was added to the medium. When glucose replaced lactate, there was a 41% reduction in the specific activity of LacZ (Table 3). These results are similar to those obtained by measuring expression of branched-chain keto acid dehydrogenase (28). When *P. putida* JS386 was grown with histidine in place of branched-chain amino acids, addition of glucose reduced *lacZ* expression by only 6%, which is probably not significant. These results strongly suggest that the repression of branched-chain keto acid dehydrogenase by glucose is due to interference with transcription of *bkdR*.

Mutations in the region of *bkdR* encoding the putative helix-turn-helix abolish transcription of the *bkd* operon. *P. putida* JS110 and JS112 are mutants which do not express any of the components of the *bkd* operon and were thought to be regulatory mutants (29). Since these mutants were created by treatment with nitrosoguanidine, they were likely to carry point mutations, possibly within *bkdR*. All of *bkdR* and the intergenic region were sequenced as described in Materials and Methods. JS110 contains a G→A transition at nt 1313, resulting in an R40Q missense mutation. This mutation would be in the recognition helix of the helix-turn-helix. *P. putida* JS112 contains a C→T transition at nt 1367 which causes a T22I mutation. This mutation would be in the first helix of the putative helix-turn-helix.

These mutations were complemented by plasmids which contained *bkdR* as the only complete reading frame. When *P. putida* JS110 and JS112 were transformed with pJRS136, which contains promoterless *bkdR* in pVLT33, growth was obtained on valine-isoleucine agar only when 0.4% IPTG was added to the medium. *P. putida* JS110 and JS112 harboring pVLT33 did not grow on valine-isoleucine agar with or without IPTG. pJRS136 also contains a partial open reading frame with sequence similarity to *glnA*. However, chromosomal mutants of *P. putida* with insertional inactivations at specific sites in *bkdR* (17) do not require even this partial open reading frame for complementation. Therefore, the phenotypes of *P. putida* JS110 and JS112 must be due to the mutations in *bkdR*.

DISCUSSION

Role of BkdR in transcription of the *bkd* operon. A model for transcriptional activation of the *bkd* operon by BkdR is presented in Fig. 6. This model predicts that L-branched-chain amino acids are the inducers of the *bkd* operon and that transcription should require millimolar L-branched-chain amino acids.

When *P. putida* is grown in a noninducing medium, the intracellular concentration of branched-chain amino acids is very low and BkdR is in the transcriptionally inactive form (Fig. 6). While this conformation does not permit transcription, three tetramers of BkdR are cooperatively bound to DNA in the region of sites I to III (Fig. 1 and 6). Recently obtained evidence suggests that BkdR binds to only one face of DNA (17a). Because of the relatively few copies per cell, a large part of BkdR is probably bound to DNA. Inactive BkdR bends DNA, but the bend angle does not result in the correct configuration for transcription. For the sake of argument, Fig. 6 shows that BkdR and RNA polymerase are not in contact.

When *P. putida* is grown in an inducing medium, the intracellular concentration of L-branched-chain amino acids approaches the millimolar range (23). L-branched-chain amino acids are the leading candidates for inducers of the *bkd* operon because of their specific effects on DNase I protection (16), BkdR conformation (14), and DNA bending demonstrated in this study. D-branched-chain amino acids and branched-chain keto acids do not cause any of these effects, although D-leucine may be an exception (17a). BkdR undergoes a conformational change to the transcriptionally active state and bends DNA such that BkdR, DNA, and RNA polymerase are in the correct configuration for transcription to take place. In Fig. 6, this is represented by BkdR and RNA polymerase coming into contact.

Substrate DNA is bent about 76° in the presence of BkdR plus L-valine or other branched-chain amino acids, compared to 92° in the presence of BkdR alone. The fact that the bend angle is less in the presence of the ligand has been observed with the LysR activators CysB (11), OccR (30), and CatR (6). The bend angle of 76° obtained in this study is shown in Fig. 6; however, RNA polymerase is also known to bend DNA (21) and the final bend angle could be greater, which would make it easier to imagine that all of bound BkdR touches both DNA and RNA polymerase.

Catabolite repression of the *bkd* operon can occur either by prevention of binding of BkdR and/or RNA polymerase to DNA or by interference with the transcription of *bkdR*, which would indirectly control expression of the *bkd* operon. The evidence presented in Table 3 shows that this control takes place by repressing expression of *bkdR*. Because there are so few copies of BkdR per cell, expression of the *bkd* operon would be sensitive to this kind of control. In any case, an additional factor, not shown in Fig. 6, must be present to cause repression of *bkdR*.

The location of the DNA-binding helix-turn-helix motif of BkdR was originally identified by sequence similarity to other

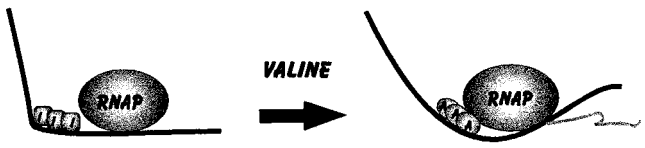

FIG. 6. Model for transcription of the *bkd* operon by BkdR. I represents the transcriptionally inactive form of BkdR, and A represents the transcriptionally active form of BkdR. RNAP is RNA polymerase.

DNA-binding proteins and to the corresponding region of Lrp (17). The mutations identified in *P. putida* JS110 and JS112, which are *bkd* negative, are located in the presumed helix-turn-helix (17). Platko and Calvo identified DNA-binding, activation, and ligand-binding domains of Lrp (22) based on the effects of several mutations. They located the DNA-binding domain at the N terminus of Lrp, which agrees with the location of the DNA-binding domain in BkdR. Circular-dichroism studies suggested that the only helical structure of BkdR was in the DNA-binding domain (14). It will be interesting to identify the mutation in *P. putida* JS326 which behaves like a regulatory mutant but does not carry a mutation in *bkdR* or in the control region for the *bkd* operon.

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REFERENCES

1. **Belev, T. N., M. Singh, and J. E. G. McCarthy.** 1992. A fully modular vector system for the optimization of gene expression in *Escherichia coli*. Plasmid **26:**147–150.
2. **Burns, G., T. Brown, K. Hatter, J. M. Idriss, and J. R. Sokatch.** 1988. Similarity of the E1 subunits of branched-chain-oxoacid dehydrogenase from *Pseudomonas putida* to the corresponding subunits of mammalian branched-chain-oxoacid and pyruvate dehydrogenases. Eur. J. Biochem. **176:**311–317.
3. **Burns, G., T. Brown, K. Hatter, and J. R. Sokatch.** 1988. Comparison of the amino acid sequences of the transacylase components of branched chain oxoacid dehydrogenase of *Pseudomonas putida*, and the pyruvate and 2-oxoglutarate dehydrogenases of *Escherichia coli*. Eur. J. Biochem. **176:**165–169.
4. **Burns, G., T. Brown, K. Hatter, and J. R. Sokatch.** 1989. Sequence analysis of the *lpdV* gene for lipoamide dehydrogenase of branched chain oxoacid dehydrogenase of *Pseudomonas putida*. Eur. J. Biochem. **179:**61–69.
5. **Calvo, J. M., and R. G. Mathews.** 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Microbiol. Rev. **58:**466–490.
6. **Chakrabarty, A. M.** 1996. Microbial degradation of toxic chemicals: evolutionary insights and practical considerations. ASM News **62:**130–137.
7. **Cui, Y., Q. Wang, G. D. Stormo, and J. M. Calvo.** 1995. A consensus sequence for binding of Lrp to DNA. J. Bacteriol. **177:**4872–4880.
8. **de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123:**17–24.
9. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158:**1115–1121.
10. **Hester, K., J. Luo, G. Burns, E. H. Braswell, and J. R. Sokatch.** 1995. Purification of active E1$\alpha_2\beta_2$ of *Pseudomonas putida* branched-chain-oxoacid dehydrogenase. Eur. J. Biochem. **233:**828–836.
11. **Hryniewicz, M. M., and N. M. Kredich.** 1994. Stoichiometry of binding of CysB to *cysJIH*, *cysK*, and *cysP* promoter regions of *Salmonella typhimurium*. J. Bacteriol. **176:**3673–3682.
12. **Huang, N., K. T. Madhusudhan, and J. R. Sokatch.** 1996. Stoichiometry of BkdR to substrate DNA in *Pseudomonas putida*. Biochem. Biophys. Res. Commun. **223:**315–319.
13. **Kim, J., C. Zweig, C. Wu, and S. Adhya.** 1989. Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. Gene **85:**15–23.
14. **Madhusudhan, K. T., N. Huang, E. H. Braswell, and J. R. Sokatch.** 1996. Binding of L-branched-chain amino acids causes a conformational change in BkdR. J. Bacteriol. **179:**276–279.
15. **Madhusudhan, K. T., G. Huang, G. Burns, and J. R. Sokatch.** 1990. Transcriptional analysis of the promoter region of the *Pseudomonas putida* branched-chain keto acid dehydrogenase operon. J. Bacteriol. **172:**5655–5663.
16. **Madhusudhan, K. T., N. Huang, and J. R. Sokatch.** 1995. Characterization of BkdR-DNA binding in the expression of the *bkd* operon of *Pseudomonas putida*. J. Bacteriol. **177:**636–641.
17. **Madhusudhan, K. T., D. Lorenz, and J. R. Sokatch.** 1993. The *bkdR* gene of *Pseudomonas putida* is required for expression of the *bkd* operon and encodes a protein related to Lrp of *Escherichia coli*. J. Bacteriol. **175:**3934–3940.

17a. **Madhusudhan, K. T., and J. R. Sokatch.** Unpublished data.

18. **Marshall, V. P., and J. R. Sokatch.** 1972. Regulation of valine catabolism in *Pseudomonas putida*. J. Bacteriol. **110:**1073–1081.
19. **Mattevi, A., G. Obmolova, J. R. Sokatch, C. Betzel, and W. G. J. Hol.** 1992. The refined crystal structure of *Pseudomonas putida* lipoamide dehydrogenase complexed with NAD+ at 2.45 Å resolution. Proteins Struct. Funct. Genet. **13:**336–351.
20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Pérez-Martin, J., F. Rojo, and V. de Lorenzo.** 1994. Promoters responsive to DNA bending: a common theme in prokaryotic gene expression. Microbiol. Rev. **58:**268–290.
22. **Platko, J. V., and J. M. Calvo.** 1993. Mutations affecting the ability of *Escherichia coli* Lrp to bind to DNA, activate transcription, or respond to leucine. J. Bacteriol. **175:**1110–1117.
23. **Roberts, C. M., and J. R. Sokatch.** 1978. Branched chain amino acids as activators of branched chain ketoacid dehydrogenase. Biochem. Biophys. Res. Commun. **82:**828–833.
24. **Rothmel, R. K., D. L. Shinabarger, M. R. Parsek, T. L. Aldrich, and A. M. Chakrabarty.** 1991. Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. J. Bacteriol. **173:**4717–4724.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
27. **Sokatch, J. R., V. McCully, J. Gebrosky, and D. J. Sokatch.** 1981. Isolation of a specific lipoamide dehydrogenase for a branched-chain keto acid dehydrogenase from *Pseudomonas putida*. J. Bacteriol. **148:**639–646.
28. **Sykes, P. J., G. Burns, J. Menard, K. Hatter, and J. R. Sokatch.** 1987. Molecular cloning of genes encoding branched-chain keto acid dehydrogenase of *Pseudomonas putida*. J. Bacteriol. **169:**1619–1625.
29. **Sykes, P. J., J. Menard, V. McCully, and J. R. Sokatch.** 1985. Conjugative mapping of pyruvate, 2-ketoglutarate and branched chain keto acid dehydrogenase genes in *Pseudomonas putida* mutants. J. Bacteriol. **162:**203–208.
30. **Wang, L., J. D. Helman, and S. C. Winans.** 1992. The *A. tumefaciens* transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. Cell **69:**659–667.
31. **Wang, Q., and J. M. Calvo.** 1993. Lrp, a global regulatory protein of *Escherichia coli*, binds co-operatively to multiple sites and activates transcription of *ilvIH*. J. Mol. Biol. **229:**306–318.