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The *bkdR* Gene of *Pseudomonas putida* Is Required for Expression of the *bkd* Operon and Encodes a Protein Related to Lrp of *Escherichia coli*

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Branched-chain keto acid dehydrogenase is a multienzyme complex which is required for the metabolism of the branched-chain amino acids in *Pseudomonas putida*. The structural genes encoding all four proteins of the *bkd* operon have been cloned, and their nucleotide sequences have been determined (G. Burns, K. T. Madhusudhan, K. Hatter, and J. R. Sokatch, p. 177–184 *in* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan [ed.], *Pseudomonas: Biotransformations, Pathogenesis, and Evolving Biotechnology*, American Society for Microbiology, Washington D.C., 1990). An open reading frame which encoded a protein with 36.5% amino acid identity to the leucine-responsive regulatory protein (Lrp) of *Escherichia coli* was found immediately upstream of the *bkd* operon. Chromosomal mutations affecting this gene, named *bkdR*, resulted in a loss of ability to use branched-chain amino acids as carbon and energy sources and failure to produce branched-chain keto acid dehydrogenase. These mutations were complemented in *trans* by plasmids which contained intact *bkdR*. Mutations affecting *bkdR* did not have any effect on transport of branched-chain amino acids or transamination. Therefore, the *bkdR* gene product must affect expression of the *bkd* operon and regulation must be positive. Mutations affecting *bkdR* could also be complemented by plasmids containing *lrp* of *E. coli*. This is the first instance of a Lrp-like protein demonstrated to regulate expression of an operon outside of *E. coli*.

Branched-chain keto acid dehydrogenase is an enzyme which is common to the metabolism of valine, leucine, and isoleucine in *Pseudomonas putida* (7) and eukaryotes (10, 30). It catalyzes the oxidative decarboxylation of branched-chain keto acids formed by transamination of branched-chain amino acids. The reaction with 2-ketoisovalerate, formed from valine, is 2-ketoisovalerate + NAD^+ + CoASH → isobutyryl coenzyme A + CO_2 + NADH + H^+. The enzyme has been characterized from several sources, including *P. putida* (36), *P. aeruginosa* (24), bovine kidney (31), rabbit liver (29), rat kidney (27), and *Bacillus subtilis* (19). In the latter organism, it functions as a combined pyruvate and branched-chain keto acid dehydrogenase. Branched-chain keto acid dehydrogenase is a multienzyme complex which is composed of four proteins, E1α, E1β, E2, and E3. The E1αβ component is the dehydrogenase and decarboxylase, the E2 component catalyzes transacylation between its lipoyl residue and coenzyme A, and the E3 component is lipoamide dehydrogenase, which catalyzes the oxidation of the lipoyl residue of E2. The structure of keto acid dehydrogenase complexes has been reviewed elsewhere (10, 23, 30). Most species seem to have a single lipoamide dehydrogenase which functions as the E3 component of pyruvate, 2-ketoglutarate, and branched-chain keto acid dehydrogenase. Pseudomonads, however, have three lipoamide dehydrogenases, including specific lipoamide dehydrogenases for pyruvate (LpdG) and branched-chain keto acid dehydrogenases (LpdV) (28).

The genes encoding the four proteins of branched-chain keto acid dehydrogenase have been cloned from *P. putida*, and their nucleotide sequences have been determined (4–6). All four genes are tightly linked and have been shown to function as a single transcriptional unit (20), designated the *bkd* operon (Fig. 1). The genes of this operon are *bkdA1* and *bkdA2*, encoding E1α and E1β respectively, *bkdB*, encoding the E2 component, and *lpdV*, encoding LpdV. Branched-chain keto acid dehydrogenase is induced when branched-chain amino or keto acids are provided as carbon sources (21). The object of the present study was to characterize the regulation of the *bkd* operon. During the course of this study, we learned that the deduced amino acid sequence of an open reading frame upstream of the translational start of the *bkd* operon (*bkdR* in Fig. 1) exhibited significant amino acid similarity to Lrp, a global transcriptional regulator of *E. coli* (40). This report is a description of the studies which shows that this protein, BkdR, is a positive activator of the *bkd* operon.

MATERIALS AND METHODS

Growth of bacteria. The bacterial strains used in this study are listed in Table 1. Valine/isoleucine medium (21), used for growth of *P. putida* PpG2, contains 0.3% L-valine and 0.1% L-isoleucine as carbon sources. Both amino acids are used as carbon and energy sources, and both are supplied since growth on valine alone is slow. *P. putida* was grown at 30°C, and *Escherichia coli* was grown at 37°C. The same basal salt mixture was used with other carbon sources at 10 mM, including L-glutamate, D-glucose, or 2-ketoisovalerate. The minimal medium for β-galactosidase assays with *E. coli* was described previously (32). The concentrations of antibiotics used to inhibit growth of *P. putida* were as follows: carbenicillin, 2 mg/ml; kanamycin, 90 μg/ml; and tetracycline, 200 μg/ml. The concentration of tetracycline used to inhibit growth of *E. coli* was 50 μg/ml.

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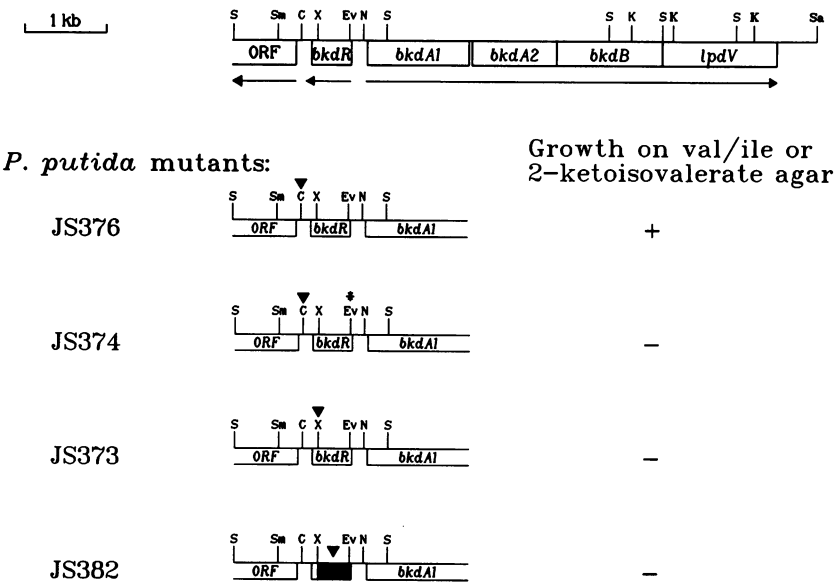

FIG. 1. (A) Organization of the *bkd* operon of *P. putida*. The structural genes are *bkdA1* encoding the E1α subunit, *bkdA2* encoding the E1β subunit, *bkdB* encoding the E2 component, and *lpdV* encoding Lpd-val, the specific lipoamide dehydrogenase for *P. putida* branched-chain keto acid dehydrogenase. ORF is a partial open reading frame encoding a protein with significant similarity to glutamine synthetase. Restriction sites: C, *Cla*I; Ev, *Eco*RV; K, *Kpn*I; N, *Not*I; S, *Sph*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I. The arrows show the direction of transcription. (B) Locations of chromosomal mutations in the strains of *P. putida* PpG2 created for this study. ▼, location of the tetracycline cassette; *, 2-bp deletion. The black rectangle in *P. putida* JS382 shows the portion of *bkdR* which has been deleted and replaced by the tetracycline cassette.

DNA manipulations and analysis. Mutants and plasmids constructed for this study are listed in Table 1. Small-scale isolation of plasmids by alkaline lysis, restriction digestions, blunting of restriction fragments, and ligations were done as described previously (34) or as instructed by the manufacturer. Transfer of plasmids from *E. coli* to *P. putida* was accomplished by using *E. coli* S17-1 (35), and *Pseudomonas* isolation agar containing appropriate antibiotics was used to inhibit growth of *E. coli*. The Wisconsin Genetics Computer Group programs (9) were used for analysis of nucleic acid and protein sequences.

Chromosomal gene replacements. The method for replacing chromosomal genes of *P. putida* by using conjugal transfer of a pUC19-based suicide plasmid was described previously (37). A tetracycline resistance determinant was used as an antibiotic marker in all of the following gene disruptions. The tetracycline resistance gene was obtained from pBR322 digested with *Eco*RI and *Sty*I, the ends were blunted, and the gene was inserted into the blunted restriction sites of the plasmid. Mutants were isolated by using *Pseudomonas* isolation agar containing 200 μg of tetracycline per ml. Double crossovers were detected by loss of resistance to carbenicillin (2 mg/ml). The insertion of the tetracycline cassette and orientation of the tetracycline gene into the chromosome were verified by restriction digestions and Southern blotting. In all cases, the tetracycline gene was transcribed in the same direction as the interrupted gene.

Mutants of *P. putida* constructed for this study are shown in Fig. 1. *P. putida* JS373 and JS374 have tetracycline cassettes cloned into the blunted *Xho*I and *Cla*I sites, respectively. *P. putida* JS376 was generated by digesting 5 μg of pJRS40 (20) with *Eco*RV followed by 5 U of exonu-

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Description	Source or reference
Bacterial strains		
P. putida		
PpG2	Wild type	I. C. Gunsalus
JS373	*bkdR* inactivated by tetracycline cassette in *Xho*I site	This study
JS374	*bkdR* inactivated by 2-bp deletion at position 23 and tetracycline cassette inserted into *Cla*I site	This study
JS376	Tetracycline cassette inserted into *Cla*I site between *bkdR* and open reading frame	This study
JS382	*Eco*RV-*Xho*I fragment of *bkdR* deleted and replaced with tetracycline cassette	This study
E. coli		
JM101	lrp^+	41
CV1304	*lrp*::Tn*10*	J. M. Calvo
CV1008	*ilvIH*::Mu dI1734 *lrp*::Tn*10*	32
S17-1	Mobilizing donor strain	35
Plasmids		
pJRS48	*bkdR* in pKT240	20
pJRS102	*bkdR* in pKRZ-1	This study
pJRS103	*lrp* in pVLT33	This study
pJRS106	*bkdA1-lacZ* fusion in pKRZ-1	This study
pKT240	Broad-host-range cloning and promoter probe vector	2
pKRZ-1	Broad-host-range cloning and promoter probe vector	33
pVLT33	$lacI^q/P_{tac}$ hybrid broad-host-range cloning vector	8

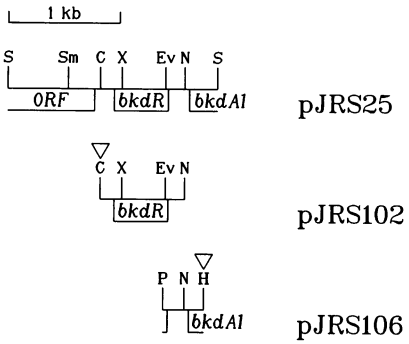

FIG. 2. DNA fragments of the *bkd* operon contained in plasmids constructed in this study. The vectors used for each plasmid are described in Table 1. ▽, location of a *lacZ* insertion. Abbreviations are as for Fig. 1.

```
 1  ATGCGCAAACTCGATCGTACCGATATCGGCATTCTCAACAGCCTGCAGGAAAACGCCCGC   60
 1  M  R  K  L  D  R  T  D  I  G  I  L  N  S  L  Q  E  N  A  R    20

61  ATCACCAACGCCGAGCTGGCACGCTCGGTCAACCTGTCGCCCACGCCCTGTTTCAACCGG  120
21  I  T  N  A  E  L  A  R  S  V  N  L  S  P  T  P  C  F  N  R    40

121 GTGCGGGCCATGGAAGAACTGGGGGTGATCCGCCAGCAGGTGACCTTGTTGTCGCCCGAG  180
41  V  R  A  M  E  E  L  G  V  I  R  Q  Q  V  T  L  L  S  P  E    60

181 GCGTTGGGGCTGGATGTGAATGTGTTCATCCATGTCAGCCTGGAAAAACAGGTAGAGCAG  240
61  A  L  G  L  D  V  N  V  F  I  H  V  S  L  E  K  Q  V  E  Q    80

241 TCGCTGCACCGCTTCGAGGAAGAAATTGCCGAACGGCCCGAGGTGATGGAGTGCTACCTG  300
81  S  L  H  R  F  E  E  E  I  A  E  R  P  E  V  M  E  C  Y  L   100

301 ATGACGGGCGACCCGGACTACCTGTTGCGGGTACTGCTGCCGAGTATCCAGGCGCTGGAG  360
101 M  T  G  D  P  D  Y  L  L  R  V  L  L  P  S  I  Q  A  L  E   120

361 CGGTTTCTCGATTACCTGACCCGGTTGCCGGGTGTGGCGAATATCCGCTCGAGTTTTGCC  420
121 R  F  L  D  Y  L  T  R  L  P  G  V  A  N  I  R  S  S  F  A   140

421 TTGAAACAGGTGCGCTACAAGACAGCCTTGCCGTTGCCGGCCAATGGCATGACCTTGCGC  480
141 L  K  Q  V  R  Y  K  T  A  L  P  L  P  A  N  G  M  T  L  R   160

481 GAATAG  486
161 E  *    162
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FIG. 3. Translated nucleotide sequence of *bkdR* from *P. putida*. $\overline{AT}$ designates the two bases deleted from *bkdR* in *P. putida* JS374.

clease III at 37°C for about 45 s. The reaction was stopped by heating at 70°C for 15 min. Three units of S1 nuclease was added and digested at room temperature for 15 min (12), and the DNA ends were blunted and ligated. The locations of the deletions were identified by DNA sequencing, and one of the clones, pJRS72, was found to have a deletion of bases 23 and 24 of *bkdR*. A tetracycline cassette was cloned into the *Cla*I site of pJRS72, and the DNA fragment was transferred to *P. putida* PpG2. *P. putida* JS382 was created by first cloning the 1,873-bp *Sph*I-*Sph*I fragment containing *bkdR* (Fig. 1) into the pUC19-based suicide plasmid. The DNA fragment between the *Eco*RV and *Xho*I sites of *bkdR* was removed, the ends were blunted, and the tetracycline resistance gene was ligated into this position.

Plasmids. Plasmids containing DNA fragments of the *bkd* operon are shown in Fig. 2. pJRS102 was constructed by isolating the *Not*I-*Cla*I fragment containing *bkdR* from pJRS25 (20) and cloning this fragment into the *Sma*I site of pKRZ-1 (33) after blunting the ends with Klenow fragment. pJRS103 was created by digesting pCV180 (11) with *Eco*RI and *Bam*HI, isolating the 500-bp fragment containing *lrp*, and ligating the fragment into pVLT33 (8) also digested with *Eco*RI-*Bam*HI. pJRS106 was constructed by digesting pJRS25 (20) with *Pst*I and *Xba*I, which removes the open reading frame (Fig. 1) and almost all of *bkdR*. The *Xba*I site is in the multiple cloning site of pJRS25. The ends of the plasmid were blunted, religated, and then digested with *Hin*cII, which cleaves *bkdA1* 129 bases downstream from the initiating methionine codon of this gene (Fig. 2). A blunt-ended fragment containing *lacZ* was obtained by digestion of pMC1871 (Pharmacia) with *Bam*HI and inserted into the *Hin*cII site of *bkdA1*, yielding pJRS106. The *lacZ* gene was oriented in the same direction as *bkdA1*. *E. coli* JM101 (41) and CV1304, kindly supplied by Joseph M. Calvo, were transformed with pJRS106.

Enzyme assays. Cell extracts were prepared as described earlier (36), using sonic oscillation and centrifugation at 90,000 × *g* for 1 h. Branched-chain keto acid dehydrogenase was measured as described previously (36). The initial rate of valine transport was determined as described previously (13), using L-[1-^{14}C]valine and 50 mM Na^+ in order to measure all transport systems and counting the radioactivity in the trichloroacetic acid precipitate. Branched-chain amino acid transaminase was measured as described elsewhere (1). The method of Miller (25) was used for the measurement of β-galactosidase activity.

RESULTS

Identification of an open reading frame encoding a protein with similarity to Lrp of *E. coli*. An open reading frame was identified in the region 5′ to *bkdA1* but on the opposite strand (*bkdR*; Fig. 1). The translated nucleotide sequence of *bkdR* is shown in Fig. 3. The gene is 486 nucleotides in length, with 161 amino acid codons plus a stop codon. The mol% G+C was 59.46, with 80.6% of the codons ending in G or C, which is typical of other genes from *P. putida*. The deduced amino acid sequence of the protein encoded by *bkdR* has 36.5% identity and 55.8% similarity to Lrp (40) of *E. coli*. For comparison, *lrp* is 495 nucleotides in length, encoding 164 amino acids. An interesting difference between the two proteins was in their pIs, 5.89 for BkdR and 9.24 for Lrp (40). The difference is due mostly to the difference in lysine residues, 10/mol for Lrp and 4/mol for BkdR. Neither protein contains tryptophan, and both are unusually high in leucine. BkdR contains 25 leucine residues (15.5 mol%), and Lrp contains 23 leucine residues (14 mol%). In contrast, the mol% leucine for all four proteins of the branched chain keto acid dehydrogenase complex is 7.7.

Willins et al. (40) pointed out that the deduced amino acid sequences of Lrp and AsnC (17) showed significant similarity to each other. AsnC is a positive regulator of *asnA*, the structural gene for asparagine synthetase A in *E. coli*. The alignment of all three of these proteins is shown in Fig. 4,

```
          1                                                  50
Lrp       MVDSKKRPGK DLDRIDRNIL NELQKDGRIS NVELSKRVGL SPTPCLERVR
BkdR      ........MR KLDRTDIGIL NSLQENARIT NAELARSVNL SPTPCFNRVR
AsnC      ......MENY LIDNLDRGIL EALMGNARTA YAELAKQFGV SPGTIHVRVE
Consensus ......... .lDr.DrgIL n.Lq.naRi. naELak.vgl SPtpc..RVr

          51                                                 100
Lrp       RLERQGFIQG YTALLNPHYL DASLLVFVEI TLN..RGAPD VFEQFNTAVQ
BkdR      AMEELGVIRQ QVTLLSPEAL GLDVNVFIHV SLE..KQVEQ SLHRFEEEIA
AsnC      KMKQAGIITG ARIDVSPKQL GYDVGCFIGI ILKSAKDYPS ALAKLES...
Consensus .me..G.I.g ...llsP..L g.dv.vFi.i .L...k..p. .l..fe....

          101                                                150
Lrp       KLEEIQECHL VSGDFDYLLK TRVPDMSAYR KLLGETLLRL PGVNDTRTYV
BkdR      ERPEVMECYL MTGDPDYLLR VLLPSIQALE RFL.DYLTRL PGVANIRSSF
AsnC      .LDEVTEAYY TTGHYSIFIK VMCRSIDALQ HVLINKIQTI DEIQSTETLI
Consensus .l.Ev.Ecyl .tGd.dyllk v..psi.Al. ..L...l.rl pgv..trt..

          151                173
Lrp       VMEEVKQSNR LVIKTR*... ...
BkdR      ALKQVRYKTA LPLPANGMTL RE*
AsnC      VLQNPIMRTI KP..... ...
Consensus vl..v...t. lp..... ...
```

FIG. 4. Alignment of the deduced amino acid sequences of Lrp, BkdR, and AsnC, using Lineup (9). The presumed helix-turn-helix is shaded.

and a potential helix-turn-helix motif is shaded. The designated region fits the criteria for a helix-turn-helix (3). The turn, position 39 in Fig. 3, corresponds to position 9 of Brennan and Mathews (3) and is usually a glycine residue; however, it is an asparagine in BkdR. Amino acid positions 38 and 40, which flank the turn, should be hydrophobic, which is the case in all four proteins. Positions 34 and 45, which correspond to positions 4 and 15 of Brennan and Mathews (3), should not be charged, and these are either hydrophobic (leucine and isoleucine) or polar (cysteine). There are no proline residues in the helices, although there are proline residues at positions 42 and 44 (Fig. 3) in both BkdR and Lrp, but these are probably at the start of the second helix, where they could be tolerated. A search of the data bases by using FastA (18) failed to uncover any other proteins with significant similarity to BkdR.

Mutations affecting *bkdR* result in loss of ability to grow in media with branched-chain amino acids as carbon sources. The phenotype of *P. putida* mutants lacking branched-chain keto acid dehydrogenase is a loss of ability to grow in media containing branched-chain amino or keto acids as carbon sources (38). Chromosomal mutations in and around *bkdR* were created to determine whether the *bkdR* gene product was involved in expression of the *bkd* operon. *P. putida* JS376, which contains a tetracycline cassette in the *Cla*I site between the open reading frame and *bkdR* (Fig. 1), grew readily on valine/isoleucine and 2-ketoisovalerate media as sole carbon sources. However, all of the mutants in which *bkdR* was interrupted (Fig. 1) failed to grow on these media. *P. putida* JS374 was created by deleting bases 23 and 24 of *bkdR* (AT in Fig. 3), resulting in a frameshift in translation of BkdR. Previous evidence indicated that the start of transcription of the *bkd* operon occurred in the region occupied by *bkdR* (20), and this mutation was created in order to cause minimal disruption of this region. *P. putida* JS373 carries a tetracycline cassette in the *Xho*I site of *bkdR*, and strain JS382 carries a tetracycline cassette inserted between the *Xho*I and *Eco*RV sites of *bkdR* (Fig. 1).

***trans* complementation of *bkdR* mutants of *P. putida* by *bkdR*.** Chromosomal mutations affecting *bkdR* in *P. putida* JS373 and JS374 resulted in loss of branched-chain keto acid dehydrogenase activity which was complemented by pJRS102. pJRS102 contains a complete copy of *bkdR* in pKRZ-1 (Table 1; Fig. 2). *P. putida* JS376, which contains an intact chromosomal *bkdR*, behaved as does the wild type, that is, produced inducible branched-chain keto acid dehydrogenase. The basal medium used in this experiment contained 10 mM L-glutamate as the carbon source, since strains JS373 and JS374 were unable to grow in valine/isoleucine medium. However, *P. putida* JS373(pJRS102) and JS374 (pJRS102) regained the ability to grow on agar with valine and isoleucine as the only carbon sources. *P. putida* JS373 and JS374 produced inducible branched-chain keto acid dehydrogenase only when carrying pJRS102. *trans* complementation by *bkdR* shows that *bkdR* encodes a protein which affects expression of branched-chain keto acid dehydrogenase. In addition, these results suggest that the identification of a transcriptional start site for the *bkd* operon inside *bkdR* should be reinvestigated (20). Either there are multiple start sites or the original observation was incorrect.

Mutations affecting *bkdR* do not affect branched-chain amino acid transport or transamination. Failure of *P. putida* JS373 and JS374 to grow on agar media containing valine/isoleucine or 2-ketoisovalerate could be due to a defect in a system which transports branched-chain amino acids and 2-ketoisovalerate, to a defect in transamination of branched-chain amino acids, or to a defect in branched-chain keto acid dehydrogenase. The defect in transport would have to affect a system which transports both branched-chain amino and keto acids; otherwise, the mutants would be able to use 2-ketoisovalerate as a carbon source. It has recently been shown that expression of the high-affinity system for transport of branched-chain amino acids in *E. coli* is regulated by Lrp and leucine, resulting in repression of the operon (11). Transport of branched-chain amino acids in *P. aeruginosa* is well characterized and is accomplished by a high-affinity system, LIV-I (15), and two low-affinity systems, LIV-II (14) and LIV-III (16). When *P. putida* JS376, JS374, and JS373 were grown in a medium with L-[1-^{14}C]valine (Fig. 5), there was no difference in the ability of these three mutants to incorporate labeled valine into trichloroacetic acid-precipitable protein. Thus, these mutants transported valine normally.

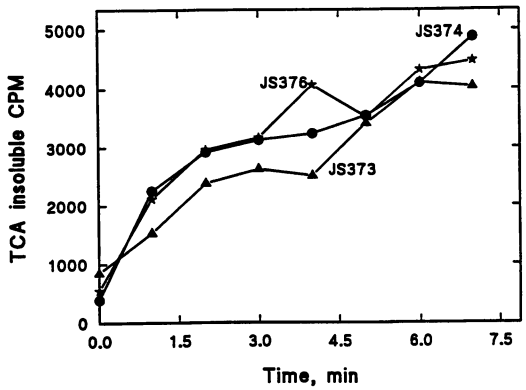

FIG. 5. *P. putida* mutants affected in expression of *bkdR* transport valine normally. *P. putida* JS373 (▲), JS374 (●), and JS376 (★) are described in Table 1. The assay for transport of branched-chain amino acids is described in Materials and Methods and measures incorporation of [1-^{14}C]valine into protein. TCA, trichloroacetic acid.

A defect in transamination should also be bypassed by the use of 2-ketoisovalerate as a carbon source; nevertheless, both transport and transamination were studied in these mutants. The *bkdR* mutants of *P. putida*, strains JS373 and JS374, produced normal amounts of transaminase (Table 3) but did not make active branched-chain keto acid dehydrogenase (Tables 2 and 3). Since Lrp has been shown to regulate expression of a number of operons in *E. coli* (26),

TABLE 2. Complementation of chromosomal *bkdR* mutations by plasmid-encoded *bkdR* in pJRS102

P. putida strain	Plasmid	Branched-chain keto acid dehydrogenase sp act (nmol of NADH formed/min/mg of protein)	
		−Val/Ile	+Val/Ile[a]
JS376	pKRZ-1	0	33
	pJRS102	0	33
JS373	pKRZ-1	0	0
	pJRS102	0	26
JS374	pKRZ-1	0	0
	pJRS102	0	28

[a] Addition of 0.3% valine and 0.1% isoleucine to basal medium with 10 mM L-glutamate.

TABLE 3. Transaminase and branched-chain keto acid dehydrogenase activities of mutants affected in the *bkdR* gene of *P. putida*

P. putida strain[a]	Sp act	
	Transaminase (nmol of keto acid formed/min/mg of protein)	Branched-chain keto acid dehydrogenase (nmol of NADH formed/min/mg of protein)
JS376	328	120
JS373	334	0
JS374	272	0

[a] Grown in medium with 10 mM glucose, 26 mM L-valine, and 8 mM L-isoleucine.

we consider it likely that the *bkdR* gene product is a positive regulator of the *bkd* operon.

Complementation of *bkdR* mutations by *lrp*. Since there is marked amino acid similarity between Lrp and BkdR, the possibility existed that these two proteins could complement each other. Two host strains affected in *bkdR* were used: *P. putida* JS382, which contains a deletion in *bkdR* between the *Xho*I and *Eco*RV sites with a tetracycline cassette inserted into this position, and *P. putida* JS373 (Fig. 1). pJRS103, which contains *lrp* cloned into the broad-host-range cloning vector pVLT33 (Table 1), was used to transform these mutants. The mutants carrying plasmid-encoded *lrp* regained the ability to grow on valine/isoleucine agar, although more slowly than the wild type. Significant complementation of the *bkdR* mutations by *lrp* was observed, as measured by branched-chain keto acid dehydrogenase activity (Table 4). In both cases, about one-fourth as much activity was obtained in the complemented mutants compared with the activity of wild-type *P. putida* PpG2. Isopropylthiogalactopyranoside (IPTG) at 0.4 mM was used to induce the expression of Lrp from pJRS103. In the absence of IPTG, *P. putida* JS373(pJRS103) and *P. putida* JS382(pJRS103) were unable to grow on valine/isoleucine agar and did not produce branched-chain keto acid dehydrogenase.

The results presented above suggested that Lrp should be able to stimulate transcription of the *bkd* operon. Plasmid pJRS106 contains a *lacZ* fusion of *bkdA1* (Table 1; Fig. 2) but does not contain *bkdR*. *E. coli* JM101 and CV1304 were transformed with pJRS106 and grown in glucose minimal medium (32) with and without leucine, and β-galactosidase expression was measured in mid-log-phase growing cells. *E. coli* JM101 and CV1304 are isogenic except for the Tn*10* insertion in *lrp* (Table 1). The results in Table 5 show that *E. coli* JM101(pJRS106) containing intact chromosomal *lrp* produced about 20 times as much β-galactosidase as did *E. coli* CV1304, which contains disrupted *lrp*. The addition of leucine stimulated the expression of β-galactosidase by about 25%. These results show that Lrp acted as a positive regulator of *bkdA1* expression.

TABLE 4. Complementation of *bkdR* mutations of *P. putida* by plasmid-encoded *lrp*

P. putida strain	Plasmid	Branched-chain keto acid dehydrogenase sp act (nmol of NADH formed/min/mg of protein)	
		−Val/Ile	+Val/Ile[a]
PpG2	None	0	20
JS382	pVLT33	0	0
JS382	pJRS103	0	6
JS373	pJRS103	0	6

[a] Addition of 0.3% valine and 0.1% isoleucine to basal medium with 10 mM L-glutamate.

TABLE 5. Stimulation of *bkdA1* transcription by *lrp* of *E. coli*

E. coli strain	Plasmid	β-Galactosidase sp act (nmol of ONPG[a] formed/min/mg of protein)	
		−Leucine	+Leucine[b]
JM101	pJRS106	34,900	44,500
CV1304	pJRS106	1,680	2,070

[a] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
[b] Addition of leucine (100 μg/ml) to glucose minimal medium.

DISCUSSION

The work presented in this report shows that BkdR probably acts as a positive activator of expression of the *bkd* operon of *P. putida*. Several types of chromosomal gene replacements which interrupted the sequence of *bkdR* all resulted in failure of *P. putida* PpG2 to grow on valine/isoleucine agar and to produced branched-chain keto acid dehydrogenase. These mutations were complemented in *trans* by *bkdR* (Table 2 and 3) and by Lrp (Table 4). Since Lrp is known to be a transcriptional regulator, BkdR must be acting similarly and regulation of the *bkd* operon by BkdR must be positive. A coeffector must be required for the action of BkdR, since earlier work showed that the induction of branched-chain keto acid dehydrogenase in *P. putida* PpG2 took place only during growth in media containing either the branched-chain amino or keto acids (21). Some genetic evidence was obtained that the effector might be one of the branched-chain keto acids, since a mutant was obtained which produced branched-chain keto acid dehydrogenase when grown in media with any of the three branched-chain keto acids but not with the branched-chain amino acids (22). Transcriptional studies to determine the nature of the effector and more clearly define the start of transcription are now in progress.

There are three proteins in the class of transcriptional regulators to which BkdR belongs, Lrp, BkdR, and AsnC (Fig. 2). Since Lrp is the best-characterized protein of this group and most is known about its action, it seems appropriate to refer to these proteins as the Lrp family of regulatory proteins. One of the most interesting features of Lrp is its activity as a regulator of several operons in *E. coli* (26). lrp is in some cases a positive regulator and in others a negative regulator; in some cases leucine is required for action by Lrp, and in other cases leucine is not required. The best-characterized function of Lrp is as the positive transcriptional activator of the *ilvIH* operon of *E. coli* (32, 39, 40). In the absence of leucine in the medium, Lrp stimulates transcription of the *ilvIH* operon, whereas exogenous leucine results in repression of the *ilvIH* operon. The action of Lrp is complex, but purified Lrp stimulated transcription from a promoter close to the translational start of *ilvI* and leucine decreased this activity under some conditions (39).

The complementation of *bkdR* mutations by Lrp was an interesting finding, particularly since complementation took place only on valine/isoleucine agar (Table 4). This finding

suggests that valine and isoleucine, as well as leucine (Table 5), can act as effectors of Lrp to stimulate expression of the *bkd* operon. At present, we do not have any evidence for regulation of any other operons by BkdR in *P. putida*, although we have only looked at growth phenotypes.

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REFERENCES

1. **Airas, R. K.** 1989. Branched-chain-amino-acid aminotransferase assay using radioisotopes. Biochim. Biophys. Acta **992**:397–399.
2. **Bagdasarian, M. M., E. Amann, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1983. Activity of the hybrid trp-lac (tac) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene **26**:273–282.
3. **Brennan, R. G., and B. W. Mathews.** 1989. The helix-turn-helix DNA binding motif. J. Biol. Chem. **264**:1903–1906.
4. **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.
5. **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.
6. **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.
7. **Burns, G., K. T. Madhusudhan, K. Hatter, and J. R. Sokatch.** 1990. Organization and regulation of the operon encoding the branched chain keto acid dehydrogenase of *Pseudomonas putida*, p. 177–184 *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
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. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
10. **Eisenstein, R. S., R. H. Miller, G. Hoganson, and A. E. Harper.** 1990. Phylogenetic comparisons of the branched chain α-ketoacid dehydrogenase complex. Comp. Biochem. Physiol. **97B**: 719–726.
11. **Haney, S. A., J. V. Platko, D. L. Oxender, and J. M. Calvo.** 1992. Lrp, a leucine-responsive protein, regulates branched-chain amino acid transport genes in *Escherichia coli*. J. Bacteriol. **174**:108–115.
12. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
13. **Hoshino, T.** 1979. Transport systems for branched-chain amino acids in *Pseudomonas aeruginosa*. J. Bacteriol. **139**:705–712.
14. **Hoshino, T., and K. Kose.** 1989. Cloning and nucleotide sequence of *braC*, the structural gene for the leucine-, isoleucine-, and valine-binding protein of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **171**:6300–6306.
15. **Hoshino, T., and K. Kose.** 1990. Cloning, nucleotide sequences, and identification of products of the *Pseudomonas aeruginosa* PAO *bra* genes, which encode the high-affinity branched-chain amino acid transport system. J. Bacteriol. **172**:5531–5539.
16. **Hoshino, T., K. Kose-Terai, and Y. Uratani.** 1991. Isolation of the *braZ* gene encoding the carrier for a novel branched-chain amino acid transport system in *Pseudomonas aeruginosa* PAO. J. Bacteriol. **173**:1855–1861.
17. **Kölling, R., and H. Lother.** 1985. AsnC: an autogenously regulated activator of asparagine synthetase A transcription in *Escherichia coli*. J. Bacteriol. **164**:310–315.
18. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
19. **Lowe, P. N., J. A. Hodgson, and R. N. Perham.** 1983. Dual role of a single multienzyme complex in the oxidative decarboxylation of pyruvate and branched chain 2-oxoacids in *Bacillus subtilis*. Biochem. J. **215**:133–140.
20. **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.
21. **Marshall, V. P., and J. R. Sokatch.** 1972. Regulation of valine catabolism in *Pseudomonas putida*. J. Bacteriol. **110**:1073–1081.
22. **Martin, R. R., J. R. Sokatch, and L. Unger.** 1973. Common enzymes of branched-chain amino acid catabolism in *Pseudomonas putida*. J. Bacteriol. **115**:198–204.
23. **Mattevi, A., A. de Kok, and R. N. Perham.** 1992. The pyruvate dehydrogenase multienzyme complex. Curr. Opin. Struct. Biol. **2**:877–887.
24. **McCully, V., G. Burns, and J. R. Sokatch.** 1986. Resolution of branched-chain oxo acid dehydrogenase complex of *Pseudomonas aeruginosa*. Biochem. J. **233**:737–742.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Newman, E. B., R. D'Ari, and R. T. Lin.** 1992. The leucine-Lrp regulon in *E. coli*: a global response in search of a raison d'etre. Cell **68**:617–619.
27. **Odessey, R.** 1982. Purification of rat kidney branched-chain oxo acid dehydrogenase complex with endogenous kinase activity. Biochem. J. **204**:353–356.
28. **Palmer, J. A., G. Burns, K. Hatter, and J. R. Sokatch.** 1992. Structure, function, and evolution of multiple lipoamide dehydrogenases of *Pseudomonas putida*, p. 239–248. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
29. **Paxton, R., and R. A. Harris.** 1982. Isolation of rabbit liver branched chain α-ketoacid dehydrogenase and regulation by phosphorylation. J. Biol. Chem. **257**:14433–14439.
30. **Perham, R. N., L. C. Packman, and S. E. Radford.** 1987. 2-Oxo acid dehydrogenase multi-enzyme complexes: in the beginning and halfway there. Biochem. Soc. Symp. **54**:67–81.
31. **Pettit, F. H., S. J. Yeaman, and L. J. Reed.** 1978. Purification and characterization of branched chain α-ketoacid dehydrogenase complex of bovine kidney. Proc. Natl. Acad. Sci. USA **75**:4881–4885.
32. **Platko, J. V., D. A. Willins, and J. M. Calvo.** 1990. The *ilvIH* operon of *Escherichia coli* is positively regulated. J. Bacteriol. **172**:4563–4570.
33. **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.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
35. **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.
36. **Sokatch, J. R., V. McCully, and C. M. Roberts.** 1981. Purification of a branched-chain keto acid dehydrogenase from *Pseudomonas putida*. J. Bacteriol. **148**:647–652.
37. **Steele, M. I., D. Lorenz, K. Hatter, A. Park, and J. R. Sokatch.**

1992. Characterization of the *mmsAB* operon of *Pseudomonas aeruginosa* PAO encoding methylmalonate-semialdehyde dehydrogenase and 3-hydroxyisobutyrate dehydrogenase. J. Biol. Chem. **267:**13585–13592.

38. **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.
39. **Willins, D. A., and J. M. Calvo.** 1992. In vitro transcription from the *Escherichia coli ilvIH* promoter. J. Bacteriol. **174:**7648–7655.
40. **Willins, D. A., C. W. Ryan, J. V. Platko, and J. M. Calvo.** 1991. Characterization of Lrp, an *Escherichia coli* regulatory protein that mediates a global response to leucine. J. Biol. Chem. **266:**10768–10774.
41. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.