JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 636–641 Vol. 177, No. 3
0021-9193/95/$04.00+0

Characterization of BkdR-DNA Binding in the Expression of the *bkd* Operon of *Pseudomonas putida*

K. T. MADHUSUDHAN, NING HUANG, AND JOHN R. SOKATCH*

Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

Received 21 July 1994/Accepted 30 November 1994

The *bkd* operon of *Pseudomonas putida* consists of the structural genes encoding the components of the inducible branched-chain ketoacid dehydrogenase. BkdR, a positive regulator of the *bkd* operon and a homolog of Lrp of *Escherichia coli* is encoded by a structural gene adjacent to, and divergently transcribed from, the *bkd* operon of *P. putida*. BkdR was purified from *E. coli* containing *bkdR* cloned into pCYTEXP1, an expression vector. The molecular weight of BkdR obtained by gel filtration indicates that BkdR is a tetramer, and the abundance of BkdR in *P. putida* was estimated to be about 25 to 40 copies of the tetramer per cell. BkdR bound specifically to the region between *bkdR* and *bkdA1*, the latter being the first gene of the *bkd* operon. One BkdR-DNA complex was observed in gel mobility shift patterns. Approximately 100 bp was protected from the action of DNase I by BkdR, and the addition of L-branched-chain amino acids enhanced the appearance of hypersensitive sites in the protected region. There are four potential BkdR-DNA binding sequences in this region based on similarity to Lrp-binding consensus sequences. Like many other transcriptional activators, BkdR regulates expression of its structural gene. DNAs from several gram-negative bacteria hybridized to a probe containing *bkdR*, indicating the presence of *bkdR*-like genes in these organisms.

Branched-chain ketoacid dehydrogenase of *Pseudomonas putida* is an inducible multienzyme complex produced by the organism grown in media with branched-chain amino or keto acids as carbon sources (15). The formation of branched-chain ketoacid dehydrogenase is repressible by glucose and NH_4^+ (27), and the effects seem to be additive. The components of branched-chain ketoacid dehydrogenase are E1 (the dehydrogenase-decarboxylase), E2 (the transacylase), and E3 (lipoamide dehydrogenase). The structural genes for these proteins are encoded by the *bkd* operon of *P. putida*, which has been cloned and whose nucleotide sequence has been determined (3–5). All four genes are tightly linked, and the operon is transcribed as a polycistronic message (5).

An open reading frame upstream of, and divergently transcribed from, the *bkd* operon, encoding a protein, BkdR (13), with 37.5% amino acid sequence identity to Lrp, the leucine-responsive regulatory protein of *Escherichia coli* (33), was found. Lrp is a global transcriptional regulator in *E. coli* (6), the action of which can be antagonized or potentiated by leucine or may be insensitive to the presence of leucine. Expression of the *ilvIH* operon of *E. coli* is regulated by Lrp and is repressible by leucine. Lrp binds to several sites in the regulatory region of the *ilvIH* operon of *E. coli* (29) and causes DNA bending (30).

Chromosomal mutations affecting *bkdR* resulted in failure to produce branched-chain ketoacid dehydrogenase, and mutations in *bkdR* were complemented in *trans* by both *bkdR* and *lrp* (13). These results suggest that BkdR acts as a positive transcriptional activator of the *bkd* operon. This article describes the cloning and overexpression of *bkdR*, purification of BkdR, and the specific binding of BkdR to the intergenic region between *bkdR* and *bkdA1*, the latter being the first structural gene of the *bkd* operon.

* Corresponding author. Phone: (405) 271-6645. Fax: (405) 271-3139. Electronic mail address: john-sokatch@uokhsc.edu.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Valine-isoleucine medium (15) 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 same basal salt mixture was used with L-glutamate at 10 mM. *P. putida* was grown with aeration at 30°C, and *E. coli* was grown with aeration at 37°C. For β-galactosidase measurements, 5-ml overnight cultures of *P. putida* JS385 or JS386 carrying plasmids listed in Table 2 were used to inoculate 200 ml of minimal medium containing glutamate with or without valine-isoleucine. The cells were grown at 30°C until an A_{600} of 0.5, harvested, and then broken by sonic oscillation as described below in "Enzymatic methods." The final concentrations of antibiotics to inhibit the growth of *P. putida* were as follows: carbenicillin, 2 mg/ml; kanamycin, 90 μg/ml; tetracycline, 200 μg/ml. The concentration of tetracycline used to inhibit the growth of *E. coli* was 50 μg/ml.

Enzymes and chemicals. Restriction endonucleases and other DNA-modifying enzymes were obtained from Promega Biotec (Madison, Wis.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The RadPrime Labeling System was obtained from Gibco BRL (Gaithersburg, Md.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) low-molecular-weight markers, blue dextran, and chromatography media were from Pharmacia Biochemicals (Piscataway, N.J.); the picoBLUE immunoscreening kit was from Stratagene (La Jolla, Calif.); yeast tRNA, lysozyme, bovine serum albumin, and soybean trypsin inhibitor type 1S were obtained from Sigma Chemical Co. (St. Louis, Mo.); GeneScreen Plus hybridization membranes, [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.). Lrp was a gift of Joseph Calvo, Cornell University, Ithaca, N.Y.

Nucleic acid isolation and analysis. Chromosomal DNA purification over cesium chloride gradients by ultracentrifugation, small-scale isolation of plasmids by alkaline lysis, restriction digestions, blunt ending of restriction fragments, DNA labelling, and ligations were done as described in reference 23. For Southern blots, about 5 μg of purified bacterial chromosome was electrophoresed in a 0.9% agarose gel, and the DNA was transferred to a GeneScreen Plus membrane according to the manufacturer's instructions. The membrane was prehybridized and then hybridized at 55°C overnight with a random primer-labelled probe consisting of the 415-bp *Pst*I-*Nae*I region of *bkdR* obtained by digestion of pJRS146 with *Pst*I, one of the *Pst*I sites being in the multiple cloning site of pJRS146 (Fig. 1). The membrane was washed at 55°C according to the manufacturer's recommendations and autoradiographed.

Construction of chromosomal *bkdR′-lacZ* translational fusions. The source of *lacZ* for the construction of *bkdR′-lacZ* translational fusions was pMC1871 (Pharmacia Biochemicals), which contains *lacZ* minus the promoter, ribosome binding site, and codons for the first eight nonessential amino acids (7). There are three unique restriction sites each at the 5′ and 3′ ends of *lacZ* which can be used to create in-frame (i.e., translational) gene fusions. The construction of *P.*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Agrobacterium tumefaciens		
A348	Wild-type octapine strain (pTiA6NC)	L. Unger
C58	Wild-type nopaline strain (pTiC58m pATC58)	L. Unger
E. coli		
DH5α	*supE44 hsdR17 deoR recA13 endA1 lacZ*ΔM15	Bethesda Research Laboratories
S17-1	Mobilizing donor strain	24
P. aeruginosa PAO	ATCC 25692	17
Pseudomonas cepacia	ATCC 17616	T. Lessie
P. putida		
PpG2	Wild type	I. C. Gunsalus
JS382	*Eco*RV-*Xho*I fragment of *bkdR* deleted and replaced with *tet*	13
JS385	*bkdR′-lacZ* fusion; *tet* inserted in the *Bst*EII site of *bkdR*	This study
JS386	*bkdR′-lacZ* fusion; *tet* inserted in the *Cla*I site	This study
Rhizobium leguminosarum	ATCC 10314	M. McInerney
Rhodobacter sphaeroides	Wild type	S. Kaplan
S. mutans	Ingbritt (subtype C)	J. J. Ferretti
Plasmids		
pCYTEXP1	Expression vector	1
pJRS104	*Sma*I-*Sph*I fragment (Fig. 1) cloned in pJRS105	This study
pJRS105	pKRZ-1 Δ*lacZ*	This study
pJRS119	*bkdR*$^+$ cloned in the *Nde*I site of pCYTEXP1	This study
pJRS146	*Nae*I-*Nae*I fragment (Fig. 1) cloned in pUC19	This study

putida JS385 began with digestion of pMC1871 with *Bam*HI, which released a 3,072-bp DNA fragment containing *lacZ*. The fragment was blunt ended and cloned into the *Eco*RV site of pJRS40, which contains *bkdR*, the first 143 bases of *bkdA1*, and 946 bases downstream of *bkdR* on a 1,872-bp fragment in pUC19 (12). This intermediate plasmid contained an in-frame *lacZ* fusion after the eighth codon of *bkdR* at the *Eco*RV site, which was verified by its DNA sequence. Double crossover mutants of *P. putida* were created by a published procedure (26) after the introduction of an *oriT* fragment from pLAFR1 (9) to facilitate transfer of plasmids to *P. putida* and a *tet* gene into the blunted *Bst*EII site of *bkdR*. This plasmid was transferred to *E. coli* S17-1 (24) by electroporation and then mated into *P. putida* PpG2 to obtain *P. putida* JS385. Double crossover mutants were Tet^r Amp^s and unable to use branched-chain amino acids as carbon sources. The insertion of the genes into the chromosome was verified by restriction digestions and Southern blotting (23).

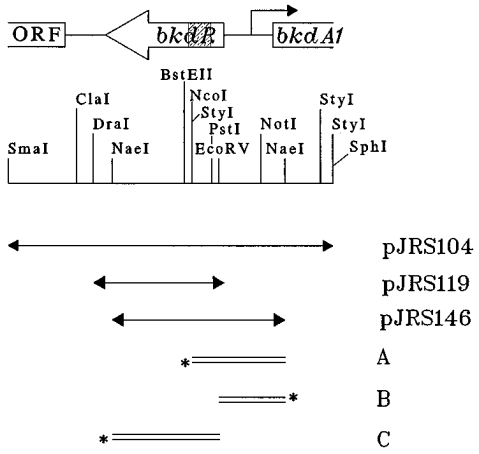

FIG. 1. Map of *bkdR*, its flanking regions, and restriction sites used in this study. *bkdA1* is the first gene of the *bkd* operon, encoding the α subunit of the E1 component; *bkdR* encodes BkdR; and the open reading frame (ORF) encodes a protein with sequence similarity to glutamine synthetase. The putative helix-turn-helix is hatched. The plasmids are described in Materials and Methods and in Table 1. A to C, DNA fragments used for gel shift and DNase I footprinting assays. Fragments A and C were labelled with ^{32}P at the 3′ end of the bottom strand; fragment B was labelled at the 3′ end of the top strand. "Top" and "bottom" refer to the DNA strands shown in this figure and in Fig. 5.

For the construction of *P. putida* JS386, pJRS40 (12) was digested with *Nco*I, and the ends were filled in. Next, the 3,072-bp *lacZ* fusion cassette described in the previous paragraph was cloned into the blunt-ended plasmid, yielding an intermediate plasmid containing a *lacZ* translational fusion after the 44th amino acid codon of *bkdR* at the *Nco*I site. Again, the construction was verified by its DNA sequence, and the remaining steps were as described for construction of *P. putida* JS385, except that in this case *tet* was inserted into the *Cla*I site of pJRS 40, immediately downstream of *bkdR*.

Plasmid constructions. pJRS104 was constructed by digesting pJRS40 (12) with *Sma*I, releasing the *Sma*I-*Sph*I fragment (Fig. 1) containing *bkdR* and the proximal portions of *bkdA1* and the open reading frame. This fragment was ligated into the *Sma*I site of pKRZ-1 (22), a promoter probe vector designed for use with gram-negative bacteria. The reporter gene in pKRZ-1 is *lacZ*, most of which was removed by digestion with *Eco*RI, yielding pJRS104. The Δ*lacZ* derivative of pKRZ-1, pJRS105, was created by digesting pKRZ-1 with *Eco*RI and religating the digest.

The *bkdR* expression plasmid pJRS119 was constructed by cloning *bkdR* generated by PCR into the *E. coli* expression plasmid pCYTEXP1 (1). The primers used were 5′-ATTTTGTCATATGCGCAAA-3′ and 5′-GCCGCGTGGGACG CC-3′, which are complementary to the *P. putida* DNA fragment cloned in pJRS40 (12) at 1,441 and 781 nucleotides (nt) (GenBank accession number, M57613). The first oligonucleotide created an *Nde*I site in the amplified product at the initiating methionine codon of *bkdR*. The 635-bp amplified product was digested with *Nde*I and *Dra*I to give a 523-bp fragment containing *bkdR*, which was cloned into the *Nde*I and *Sma*I sites of pUC18 (28). This intermediate construct helped to create an *Sph*I site for the final cloning of the PCR product into pCYTEXP1. pJRS119 was constructed by digesting the pUC18-intermediate construct with *Nde*I and *Sph*I and transcloning the insert into the same sites

of pCYTEXP1. This plasmid construct was transformed into *E. coli* DH5α by electroporation.

pJRS146 was constructed by digesting pJRS40 (12) with *Nae*I, and the resulting 705-bp fragment containing part of *bkdR*, the *bkdR-bkdA1* intergenic region, and the 5′ terminus of *bkdA1* (Fig. 1) was cloned into the *Hin*cII site of pUC19, with *bkdA1* in the same orientation as *lacZ*.

Gel mobility shift assays. Fragment A (Fig. 1), containing the *bkdR-bkdA1* intergenic region, was prepared by digesting pJRS146 with *Sty*I, located in *bkdR*, and *Eco*RI, located in the multiple cloning site, to give a 378-bp fragment which includes the *Sty*I-*Nae*I region. Fragment A was labelled at the 3′ end of the *Sty*I site with [α-^{32}P]dCTP and Klenow fragment, which labelled the bottom strand (Fig. 1) (top strand and bottom strand are defined in the legend to Fig. 1). Fragment C (Fig. 1), also used for gel mobility shifts assays, was prepared by digesting pJRS146 with *Eco*RV, located in *bkdR*, and *Hin*dIII, located in the multiple cloning site. Fragment C was 435 bp in length and included the *Nae*I-*Eco*RV region of *bkdR*. Fragment C was labelled at the 3′ end of the bottom strand with [α-^{32}P]dCTP and Klenow fragment. All labelled fragments were purified by electrophoresis through 5% polyacrylamide gels before use.

Gel mobility shift assays were performed with a 5% polyacrylamide gel in 1× TAE (40 mM Tris-acetate, 2 mM EDTA) buffer. The 1× binding buffer contained 20 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 50 mM NaCl. Protein-DNA binding was initiated by mixing the binding buffer, salmon sperm DNA (0.5 μg), and branched-chain amino or keto acids (50 mM unless otherwise noted) with the labelled DNA in a total volume of 10 μl, and protein was added as described in the legend to Fig. 3. The reaction mixtures were incubated at room temperature for 5 min before loading. Electrophoresis was conducted for 1.5 h at 100 V, and the gels were dried before autoradiography.

DNase I footprinting. Two fragments were used for DNase I footprinting. The first was fragment A (described above), labelled at the 3′ end of the bottom strand (Fig. 1). The second, fragment B, 217 bp, was prepared by digesting pJRS146 with *Eco*RV (located in *bkdR*) and *Xba*I (located in the multiple cloning site), releasing the *Eco*RV-*Nae*I region shown in Fig. 1. The 3′ end of the *Xba*I site was labelled with Klenow fragment and [α-^{32}P]dCTP, which labelled the top strand (Fig. 1). The reaction mixture of 20 μl contained 50,000 Cerenkov counts of labelled DNA, 1.2 μg of poly(dI-dC) in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 50 mM NaCl, 50 μg of bovine serum albumin per ml, and 270 nM BkdR and was incubated for 10 min at room temperature. When branched-chain amino acids or α-ketoisovalerate was present, the concentration was 50 mM. Next, 2 μl of 100 mM $MgCl_2$–30 mM $CaCl_2$ and 10 to 20 mU of DNase I (Boehringer Mannheim) in 1 μl were added, and the mixture was incubated for 1 min at room temperature. The reaction was terminated by the addition of 60 μl of stop buffer consisting of 150 mM NaCl, 0.7% SDS, 15 mM EDTA, and 100 μg of tRNA per ml. The samples were extracted with phenol-chloroform, precipitated with ethanol, and washed with 70% ethanol before the DNA pellet was solubilized by addition of 10 μl of 47% formamide, 10 mM EDTA, 0.05% bromphenol blue, and 5% xylene cyanol. The samples were heated at 90°C for 1 min and electrophoresed on a 6% DNA-sequencing gel containing 7 M urea. The nucleotides protected by DNase I were identified by electrophoresing chemical sequencing reactions (G, G+A, and C) of DNA probes alongside DNase I footprinting reactions as described in reference 16.

Enzymatic methods. For measurement of the activity of *bkdR-lacZ* fusions, the cells were harvested at mid-log phase and suspended in 0.1 M sodium phosphate buffer, pH 7.5, containing 5 mM β-mercaptoethanol. The cells were sonicated in an ice bath and centrifuged at 90,000 × *g* at 4°C for 1 h. The protein content of the cell extracts was estimated by the biuret method (11) using bovine serum albumin as a standard. The β-galactosidase activity of the extracts was measured by the method of Miller (18).

Purification of BkdR. For the purification of BkdR, 2 liters of L broth containing 200 μg of ampicillin per ml was inoculated with 20 ml of an overnight culture of *E. coli* DH5α(pJRS119) grown in the same medium. The culture was grown at 30°C with aeration until an A_{600} of 0.8. The temperature was quickly shifted to 42°C, and the culture was grown at this temperature for 3 h. The cells were harvested and then suspended in 18 ml of TMN buffer (20 mM Tris-HCl, pH 7.5, containing 0.05% β-mercaptoethanol and 0.02% NaN_3). The cell suspension was placed in an ice bath, and the cells were broken by sonic oscillation (25) and then centrifuged for 1 h at 90,000 × *g*. The protein concentration of the crude extract was estimated by the biuret method (11). All of the purification procedures were done at 4°C, starting with 250 to 300 mg of protein, which was loaded on a DEAE Sepharose CL-6B column (2 by 17 cm) equilibrated with TMN buffer containing 0.1 M NaCl. Unbound proteins were eluted with 100 ml TMN buffer plus 0.1 M NaCl, and bound proteins were separated by a linear gradient of 0.1 to 0.3 M NaCl (400 ml) in TMN buffer. Purification of BkdR was followed by SDS-PAGE, looking for the characteristic protein band at about 20 kDa. Fractions of 4 ml were collected, and the fractions containing BkdR were pooled. The protein content of column fractions was determined by the dye binding assay (2) using the Bio-Rad (Hercules, Calif.) microassay procedure with bovine serum albumin as a standard. The pool from DEAE Sepharose CL-6B chromatography, containing 80 to 100 mg of protein, was loaded onto a heparin Sepharose CL-6B column (1.6 by 11 cm), and unbound proteins were eluted by washing with 5 column volumes of TMN buffer containing 10 mM $MgCl_2$ and 0.1 M NaCl. Bound proteins were separated by a gradient of 0.1 to 0.4 M NaCl in 150 ml of TMN buffer plus 10 mM $MgCl_2$ and 0.1 M NaCl. Fractions of 2 ml were collected, and the fractions containing BkdR were pooled and dialyzed overnight against TMN buffer containing 2% glycerol, causing precipitation of BkdR. The retentate was centrifuged at 10,000 rpm for 20 min in a Sorvall RC5C centrifuge at 12,000 × *g*. The pellet was redissolved in 10 μM Tris-HCl (pH 8.0)–1 μM EDTA–0.005% β-mercaptoethanol–0.2 M NaCl–50% glycerol and stored at −70°C until used. The yield was 2 to 3 mg of purified BkdR from 250 mg of crude extract.

The N-terminal amino acid sequence of BkdR was determined by the Molecular Biology Resource Facility of the University of Oklahoma Health Sciences Center.

The molecular weight of BkdR was estimated by gel filtration chromatography using a Sephadex G-150 column (1.0 by 25 cm) (particle size, 40 to 120 μm) equilibrated with 20 mM sodium phosphate, pH 8.0, containing 10% glycerol, 200 mM NaCl, 0.005% β-mercaptoethanol, 1 mM EDTA, and 0.02% NaN_3. About 100 μg of BkdR or standard proteins was loaded onto the column in a volume of 60 to 100 μl of buffer and eluted at a flow rate of 20 ml/h at room temperature. Fractions of 0.7 ml were collected, and the protein contents of the fractions were monitored by measuring the A_{280} (11) or by the dye binding method (2). Molecular weight standards used were blue dextran, Lpdval of *P. putida* (98,000) (5), bovine serum albumin (67,000), soybean trypsin inhibitor (20,000), and lysozyme (14,000).

The absorption spectrum of BkdR was obtained by diluting purified BkdR to 0.1 mg/ml in 1 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.2 mM sodium chloride–0.005% β-mercaptoethanol–10% glycerol. The absorption spectrum from 240 to 320 nm was scanned and recorded by a Gilford Response UV-vis spectrophotometer.

Western blots (immunoblots). Rabbit polyclonal antibodies were prepared by Rockland, Inc. (Gilbertsville, Pa.), by injecting 15 μg of purified BkdR into a New Zealand White rabbit with Freund's complete adjuvant, and two boosters of 15 μg each were given in Freund's incomplete adjuvant at 2-week intervals. Serum was obtained on the fourth day following the second booster and was preabsorbed with 75 μg of *P. putida* JS382 protein per μg of antiserum protein. *P. putida* JS382 is a *bkdR* deletion mutant (Table 1).

For Western blots, proteins were fractionated on a 12% separating gel and electrophoretically transferred to a BA-S supported nitrocellulose transfer membrane (Schleicher and Schuell, Keene, N.H.). Detection of BkdR was accomplished with an ECL Western blotting analysis system from Amersham (Arlington Heights, Ill.) with the following modifications. The membrane was blocked with 5% blocking reagent in Tris-buffered saline with 0.5% Tween 20 (BTBST) at 20°C for 1 h before incubation with primary and secondary antibodies. The preabsorbed primary antibody was diluted with 25 μl of BTBST per μg of antibody protein and incubated with the membrane at 20°C for 1 h. The secondary antibody was goat anti-rabbit serum conjugated with horseradish peroxidase diluted 1:2,000 (vol/vol) with BTBST and incubated at 20°C for 1 h. Washing and detection were as described in the manufacturer's protocol with exposure times of 20 to 30 min.

RESULTS

Purification and properties of BkdR. Expression of *bkdR* in *E. coli* DH5α was achieved with pJRS119, which contains *bkdR* cloned in pCYTEXP1 and whose construction is described in Materials and Methods. pCYTEXP1 is an *E. coli* expression vector which contains the $\lambda P_R P_L$ promoter and the *atpE* translation initiation region and whose expression is controlled by the temperature-sensitive λ repressor *cIts*857 (1). BkdR was present in both the soluble and the pellet fractions of the induced cultures, but only soluble BkdR was purified (Fig. 2), because insoluble BkdR was inactive. Induction of the cultures beyond 3 h did not significantly increase the yield of *bkdR* in the soluble fraction. The final step was precipitation of BkdR by dialysis of the pooled fractions obtained from heparin Sepharose CL-6B chromatography, which removed additional high-molecular-weight contaminants. The final product was biologically active in gel mobility shift assays. The sequence of the first 10 N-terminal amino acids of purified BkdR was Met Arg Lys Leu Asp Arg Thr Asp Ile Gly, which agreed with the predicted amino acid sequence (13).

The UV spectrum of BkdR (not shown) was similar to that of Lrp (33), with an absorption maximum at 278 nm with a series of ripples in the region of 256 to 270 nm, which are characteristic of phenylalanine (32). The deduced amino acid sequence of BkdR lacks tryptophan but contains 2.48 mol%

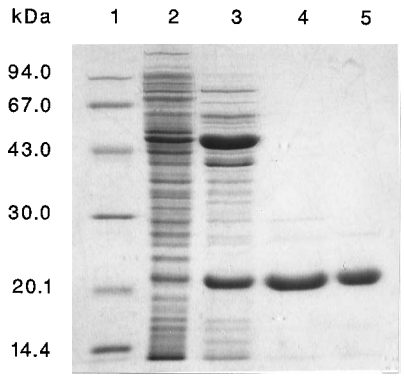

FIG. 2. Expression, purification of BkdR, and SDS-PAGE analysis of purified fractions. The samples were electrophoresed in a 12% separating gel and stained with Coomassie brilliant blue R250. Lane 1, molecular weight markers in descending molecular weight order: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin; lane 2, cell extract of *E. coli* DH5α(pJRS119), 14 μg; lane 3, DEAE Sepharose pooled fractions, 7 μg; lane 4, pooled fractions from a heparin Sepharose CL-6B column, 2.5 μg; lane 5, precipitated and redissolved BkdR from the heparin Sepharose CL-6B pool, 1.5 μg.

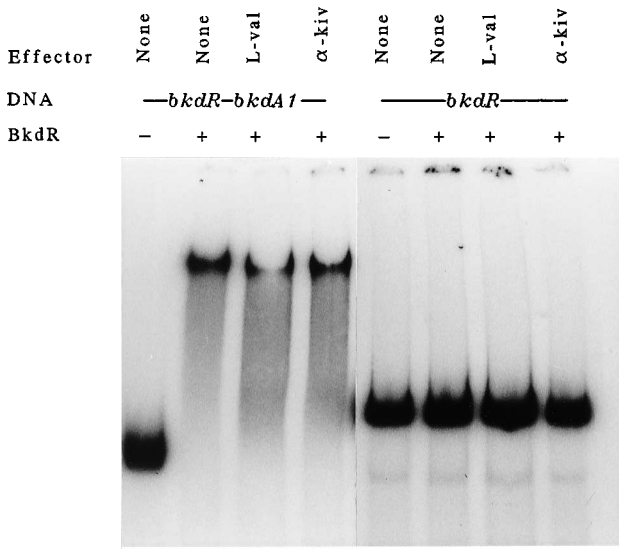

FIG. 3. Specific binding of BkdR to the *bkdR-bkdA1* intergenic region. Twenty-three nanograms of ^{32}P-labelled fragment A, which contains the *bkdR-bkdA1* intergenic region (Fig. 1) or ^{32}P-labelled fragment C, which contains only the *bkdR* coding sequence (Fig. 1), and 109 nM BkdR were incubated at room temperature and then electrophoresed in 5% polyacrylamide gel. The concentration of branched-chain amino acids or α-ketoisovaleric acid (α-kiv) was 50 mM.

tyrosine and 3.11 mol% phenylalanine (13). The molar extinction coefficient of BkdR was estimated to be 5,240 by the PeptideSort program of the Genetics Computer Group sequence analysis program (8). The protein concentration of BkdR estimated from the A_{278} was 1.7 times that obtained by the Bio-Rad microassay procedure.

The molecular weight of BkdR was estimated to be 79,400 by gel filtration chromatography. BkdR was eluted after LpdV (M_r, 98,000) and before bovine serum albumin (M_r, 67,000). The estimated molecular weight is about four times the computed molecular weight of the monomer, 18,350 (13). In comparison, Lrp is a dimer in solution (33).

Abundance of BkdR in *P. putida*. BkdR could not be detected in extracts of *P. putida* PpG2 by conventional Western blots. However, when enhanced chemiluminescence was used, it was detectable in extracts of *P. putida* PpG2 at about 2 to 3 ng/100 μg of protein. No BkdR was detectable in extracts of *P. putida* JS382, which is a *bkdR* deletion mutant (13). Assuming 155×10^{-15} g of protein per cell (19) and a calculated molecular weight of 73,400 (or four times the monomer molecular weight of 18,350), there are about 25 to 40 copies of the tetramer per cell, depending on whether the protein concentration is estimated by the dye binding method or extinction coefficient. Assuming 6.7×10^{-13} g of water per cell (19), the concentration of BkdR inside the cell is about 10^{-10} M.

BkdR binds specifically to the *bkdR-bkdA1* intergenic region. Incubation of purified BkdR with fragment A, which includes the *bkdR-bkdA1* intergenic region (Fig. 1), resulted in retardation of the DNA fragment due to binding of BkdR. Incubation of BkdR with fragment C, which includes only *bkdR* (Fig. 1), did not result in retardation, demonstrating that BkdR binds specifically to the *bkdR-bkdA1* intergenic region. Only one band was seen in the shifted region, suggesting that only one complex was formed by BkdR and fragment A. Neither L-valine nor α-ketoisovalerate had any effect on the mobility or the number and size of complexes formed with BkdR (Fig. 3). In separate experiments, it was determined that up to 200 mM L-leucine and L-isoleucine also had no apparent effect on mobility and number of complexes formed between DNA and BkdR.

Because *lrp* complemented chromosomal mutations in *bkdR* (13), there was reason to believe that Lrp should also bind to the region controlling expression of the *bkd* operon of *P. putida*. In separate experiments not shown here, it was found that Lrp also bound specifically to the *bkdR-bkdA1* intergenic region. In this case, two Lrp-DNA complexes were formed with DNA; however, larger amounts of Lrp or the addition of as little as 15 mM L-leucine caused the formation of a single complex.

DNase I protection studies. The region of DNA protected from the action of DNase I by BkdR extended from about nt 1420 to 1520 (Fig. 4 and 5), numbered as in reference 12. The pattern with BkdR alone included a distinct hypersensitive site at about nt 1453 on both the top and the bottom strands (Fig. 4). The addition of L-valine or L-leucine to the incubation mixture enhanced the appearance of the hypersensitive site at nt 1453 and produced additional sites at about nt 1485 and 1495 on both strands of DNA, although the nt 1485 site was barely visible on the bottom strand (Fig. 4). The footprint of BkdR in the presence of α-ketoisovalerate (Fig. 4) and D-valine (data not shown) was virtually identical to that of BkdR alone. These results suggest that branched-chain amino acids have an effect on binding of BkdR to DNA and possibly on the expression of the *bkd* operon. The footprint of Lrp was similar to that of BkdR, except that the hypersensitive sites were not as pronounced.

BkdR autoregulates *bkdR* expression. Many transcription factors, which are either positive (21) or negative (14) regulators, including Lrp (31), repress expression of their own structural genes. For comparison, expression of *bkdR* was studied with *P. putida* JS385 and JS386, which contain chromosomal *lacZ* translational fusions inserted into codons 9 and 45, respectively, of *bkdR* (Table 2). These mutants of *P. putida* were grown in minimal media containing 10 mM L-glutamate with or without branched-chain amino acids. When *bkdR* was supplied in *trans* on plasmid pJRS104, there was a reduction of β-galactosidase activity by 74 to 86% in both strains regardless of the medium, demonstrating autorepression of *bkdR* expression

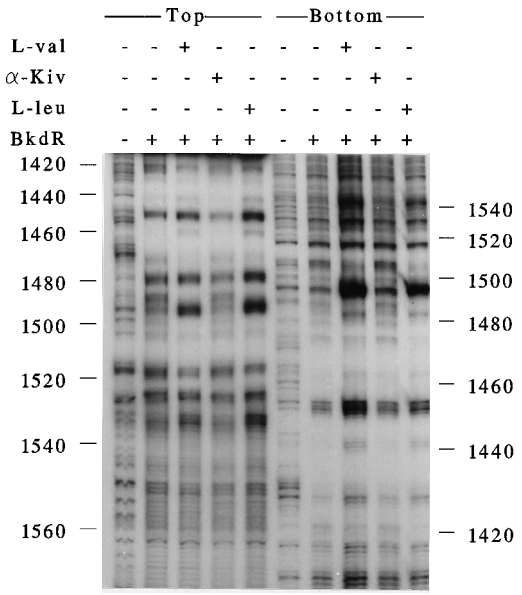

FIG. 4. DNase I footprinting of fragment B (Fig. 1), labelled at the 3′ end of the top strand of the *bkdR-bkdA1* intergenic region, and of fragment A, labelled at the 3′ end of the bottom strand. The labelled fragments were incubated with BkdR and treated with DNase I, and the digest was fractionated on denaturing acrylamide gels as described in Materials and Methods. The reaction mixtures contained 50 mM branched-chain amino acids or 50 mM α-ketoisovalerate (α-Kiv).

(Table 2). The β-galactosidase activities of both strains were 15 to 20% lower in extracts prepared from cells grown with branched-chain amino acids than in extracts from cells grown in minimal medium with L-glutamate only (Table 2). The data in Table 2 are the averages for two separate experiments. The BkdR contents of cell extracts of *P. putida* JS385 and JS386 carrying pJRS104 were about 4 to 6 ng/100 μg of protein as estimated by the ECL technique and using the dye-binding method to calibrate the concentration of purified BkdR. Therefore, the BkdR content of these cells was about 50 to 75 tetramers per cell, on the basis of the same approximations employed to calculate the abundance of BkdR in *P. putida* PpG2. No BkdR was detectable in extracts of *P. putida* JS385 or JS386 carrying pJRS105, the cloning vector minus *bkdR*.

***bkdR*-like genes are present in other gram-negative bacteria.** The existence of Lrp homologs has been reported only for *E. coli* and *P. putida*, and it was of some interest to see if other bacteria contain similar genes. Chromosomal DNAs from several species were isolated and tested for the presence of *bkdR*-like genes by Southern hybridization (Fig. 6). Hybridization signals were obtained with DNAs from several gram-negative bacteria but not with DNA from the gram-positive *Streptococcus mutans*. In a separate experiment a chromosomal digest of *P. putida* DNA with *Pst*I showed one intense band, suggesting that there is a single copy of *bkdR* on the chromosome. Chromosomal DNA of *Pseudomonas aeruginosa* showed the strongest signal of those tested.

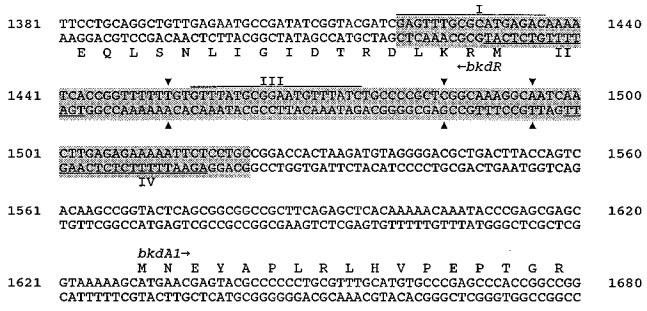

FIG. 5. Nucleotide sequence of the *bkdR-bkdA1* intergenic region showing regions of protection by BkdR against DNase I. The protected nucleotides are shaded, and the hypersensitive sites are indicated (arrowheads). Potential BkdR-DNA binding sites are designated I to IV.

TABLE 2. Effect of *bkdR*$^+$ on expression of chromosomal *bkdR′-lacZ* translational fusions

P. putida strain[a]	Plasmids	β-Galactosidase (sp act)[b]	
		Basal medium[c]	Basal medium + Val-Ile[d]
JS385	None	71	57
JS385	pJRS105/pKRZ-1 Δ*lacZ*	154	128
JS385	pJRS104/pJRS105 *bkdR*$^+$	40	30
JS386	None	249	222
JS386	pJRS105/pKRZ-1 Δ*lacZ*	557	402
JS386	pJRS104/pJRS105 *bkdR*$^+$	84	82

[a] Both strains are *bkdR′-lacZ*.
[b] In micromoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milligram of protein. The data are averages for two experiments.
[c] Containing 10 mM L-glutamate.
[d] Final concentrations: Val, 0.3%; Ile, 0.1%.

DISCUSSION

Comparison of potential BkdR binding sites with Lrp consensus sequences. Wang and Calvo (29) identified six binding sites for Lrp in the region upstream of the *ilvIH* operon and arrived at the consensus sequence binding site shown in Fig. 7. Rex et al. (20) identified the Lrp operator for the *tdh* operon of *E. coli* and compared this sequence with sequences of upstream regions of other operons known to be regulated by Lrp, including *ilvIH*, and their consensus sequence is also shown in Fig. 7. There are four sequences (I to IV) in the BkdR-protected region which are similar to the consensus sequences in

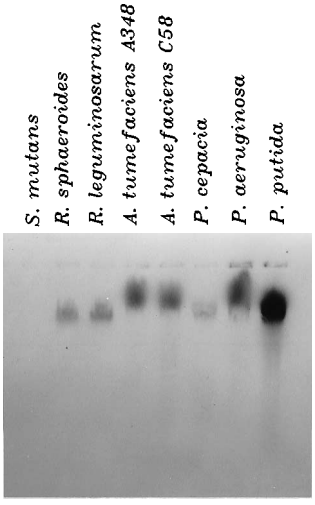

FIG. 6. Southern blots of chromosomal DNAs of gram-negative bacteria probed with *bkdR*. About 5 μg of purified chromosomal DNA was electrophoresed in agarose gels and hybridized with a 415-bp *Pst*I-*Nae*I fragment of *bkdR* as described in Materials and Methods.

AGAATTTTATTCT	Wang and Calvo (1993)
TTTATTCtNaAT	Rex *et al.* (1991)
GAGTTTGCGCATGAGAC	I
TGATTTTGTCTCAT	II
GTTTATGCGGAATGTTTAT	III
AGAATTTTTCTCTCT	IV

FIG. 7. Comparison of putative BkdR and Lrp binding sites. The direct repeats of site III are underlined.

references 29 and 20. All sequences contain at least three thymine residues. Sequence IV has the strongest similarity to the Lrp consensus sequences. Sequences I (top strand) and II (bottom strand) overlap the 5′ end of *bkdR* and could play a role in repression of *bkdR* expression (Table 2). Sequence III, on the top strand of Fig. 5, contains a direct repeat shown in Fig. 7. The *bkdR-bkdA1* intergenic region is unusually low in G+C for *P. putida*, and this low G+C content could play a role in transcriptional regulation by facilitating DNA melting and/or bending. Bergey's manual states that the average for *P. putida* is 62.5 mol% G+C (10). The intergenic region between *bkdR* and *bkdA1* is only 50 mol% G+C compared with 65.3% for all four *bkd* structural genes.

Role of BkdR in the expression of the *bkd* operon. All of the *bkdR* mutants (13) seem to be affected only in expression of the *bkd* operon, and so far there is no evidence that BkdR is a global regulator in *P. putida*. In addition, the low copy number of about 25 to 40 tetramers of BkdR per cell, in contrast to the copy number of about 3,000 dimers of Lrp per cell (33), supports this conclusion. The earlier genetic data (13) showing that *bkdR* null mutants fail to express the *bkd* operon demonstrated that BkdR is a positive transcriptional regulator of the *bkd* operon. The present biochemical studies show that the protein encoded by *bkdR* binds specifically to the *bkdR-bkdA1* intergenic region. Like many other transcriptional activators, BkdR autorepresses expression of its own structural gene (Table 2). There is clearly an effect of L-branched-chain amino acids in the DNase I protection experiments resulting in the enhancement of hypersensitive sites. The significance of this phenomenon in expression of the *bkd* operon is currently being studied. Expression of the *bkd* operon of *P. putida* takes place in media with branched-chain amino acids or branched-chain keto acids as the carbon sources (15). However, it was not possible to tell from this early study which compounds were the true inducers. The effect of branched-chain amino acids on DNase I protection studies and the failure of α-ketoisovalerate to influence the pattern of protection favors a role for branched-chain amino acids in this process. Factors other than BkdR are probably involved in expression of the *bkd* operon to account for repression by glucose and ammonium ion (27).

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grant DK-21737.

We thank Joe Calvo for the purified Lrp and for many helpful discussions.

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. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **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.
4. **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 dehydrogenase of *Escherichia coli*. Eur. J. Biochem. **176:**165–169.
5. **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.
6. **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.
7. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–308.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
10. **Krieg, N. R., and J. G. Holt.** 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
11. **Layne, E.** 1957. Spectrophotometric and turbidometric methods for measuring proteins. Methods Enzymol. **3:**447–454.
12. **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.
13. **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.
14. **Maloy, S., and V. Stewart.** 1993. Autogenous regulation of gene expression. J. Bacteriol. **175:**307–316.
15. **Marshall, V. P., and J. R. Sokatch.** 1972. Regulation of valine catabolism in *Pseudomonas putida*. J. Bacteriol. **110:**1073–1081.
16. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
17. **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.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell. Sinauer Associates, Sunderland, Mass.
20. **Rex, J. H., B. D. Aronson, and R. L. Somerville.** 1991. The *tdh* and *serA* operons of *Escherichia coli*: mutational analysis of the regulatory elements of leucine-responsive genes. J. Bacteriol. **173:**5944–5953.
21. **Rothmel, R. K., T. L. Aldrich, J. E. Houghton, W. M. Coco, L. N. Ornston, and A. M. Chakrabarty.** 1990. Nucleotide sequencing and characterization of *Pseudomonas putida catR*: a positive regulator of the *catBC* operon is a member of the LysR family. J. Bacteriol. **172:**922–931.
22. **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.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
24. **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.
25. **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.
26. **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.
27. **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.
28. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
29. **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.
30. **Wang, Q., and J. M. Calvo.** 1993. Lrp, a major regulatory protein in *Escherichia coli*, bends DNA and can organize the assembly of a higher-order nucleoprotein structure. EMBO J. **12:**2495–2501.
31. **Wang, Q., J. Wu, D. Friedberg, J. Plakto, and J. M. Calvo.** 1994. Regulation of the *Escherichia coli lrp* gene. J. Bacteriol. **176:**1831–1839.
32. **Wetlaufer, D. B.** 1962. Ultraviolet spectra of proteins and amino acids. Adv. Protein Chem. **17:**303–390.
33. **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.