JOURNAL OF BACTERIOLOGY, Oct. 1990, p. 5655–5663
0021-9193/90/105655-09$02.00/0

Vol. 172, No. 10

Transcriptional Analysis of the Promoter Region of the *Pseudomonas putida* Branched-Chain Keto Acid Dehydrogenase Operon

K. T. MADHUSUDHAN, GANG HUANG, GAYLE BURNS, AND J. R. SOKATCH*

Department of Biochemistry and Molecular Biology, The University of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, Oklahoma 73190

Received 20 April 1990/Accepted 11 July 1990

Branched-chain keto acid dehydrogenase is a multienzyme complex produced by *Pseudomonas putida* when it is grown in a minimal medium containing branched-chain amino acids. A 1.87-kilobase (kb) DNA fragment was cloned and sequenced which contained 0.24 kb of the E1α structural gene and 1.6 kb of upstream DNA. There were 854 base pairs (bp) of noncoding DNA upstream of *bkdA1*, the first gene of the *bkd* operon, and 592 bp between the transcriptional and translational starts. The G+C content of the noncoding region was 56.7% compared with 65.2% for all the structural genes of the operon. A partial open reading frame was found on the strand opposite that of the *bkd* operon beginning at base 774. When the *bkd* promoter was cloned into the promoter probe vector pKT240, streptomycin resistance was obtained in *P. putida* but not *Escherichia coli* with the promoter in both orientations, which indicates either that the *bkd* promoter is bidirectional or that there are two promoters in this region. A series of ordered deletions on both sides of the proposed site of the start of transcription revealed that almost 700 bp upstream of the start of translation were required for expression. Streptomycin resistance was also obtained in an *rpoN* mutant of *P. putida* KT2440 containing constructs with the intact *bkd* promoter, indicating that the *bkd* operon does not require the *rpoN* sigma factor for expression. Another construct containing the *bkd* promoter, *bkdA1*, and *bkdA2* in pKT240 was used to transform *P. putida* JS113, a mutant which was unable to produce the E1 subunits of the branched-chain keto acid dehydrogenase. In this case, very high inducible expression of the *bkd* operon was obtained.

Branched-chain keto acid dehydrogenase is a multienzyme complex which catalyzes the oxidation of branched-chain keto acids formed by the transamination of branched-chain amino acids (25). This enzyme is induced in *Pseudomonas putida* and *Pseudomonas aeruginosa* by growth in media containing the branched-chain amino acids or branched-chain keto acids, the latter being the true inducers (20). Branched-chain keto acid dehydrogenase purified from *P. putida* or *P. aeruginosa* consists of three components, E1, E2, and E3 or LPD-Val. The E1 component consists of two nonidentical subunits, E1α and E1β, and catalyzes the oxidative decarboxylation of the keto acid substrates. The E2 component contains covalently bound lipoic acid which is reduced by E1 and to which the acyl group becomes attached. The E3 component of *Pseudomonas* branched-chain keto acid dehydrogenase is a specific lipoamide dehydrogenase named LPD-Val since it is induced in media containing valine or other branched-chain amino acids (21, 26). The structural genes of the branched-chain keto acid dehydrogenase operon from *P. putida* were cloned, and the nucleotide sequence was determined (4–6, 28). These tightly linked genes were expressed from a single polycistronic message which was produced in media containing branched-chain amino acids (6). This message was just over 1 kilobase (kb) longer than the coding region for the structural genes, which means that there was enough room for another gene or a long leader sequence. Here, we report the nucleotide sequence of the region upstream of the branched-chain keto acid dehydrogenase structural genes, the identification of the transcriptional start, and the expression of the *bkd* operon from the *bkd* promoter.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and culture conditions. The strains, plasmids, and phage used in this study are described in Table 1. The growth conditions and media were described earlier (28, 29). *Pseudomonas* isolation agar was from Difco Laboratories (Detroit, Mich.). RNA was prepared from *P. putida* grown in a minimal medium with either 0.3% valine and 0.1% isoleucine (valine-isoleucine medium) (29) or 10 mM glucose as the sole carbon source or in L broth (17). GASV medium was used for mutants affected in keto acid dehydrogenases, including branched-chain keto acid dehydrogenase, and contains 10 mM glucose, 2 mM acetate, 2 mM succinate, 0.3% L-valine, and 0.1% L-isoleucine (28). GAS medium is similar except that valine and isoleucine are omitted. When antibiotic supplements were added, the final concentrations were ampicillin, 200 μg/ml; kanamycin, 90 μg/ml; and carbenicillin, 2,000 μg/ml.

Enzymes and chemicals. Restriction endonucleases and other DNA enzymes were obtained from Promega Biotec (Madison, Wis.) or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The [γ-^{32}P]ATP, [α-^{32}P]dCTP, and [α-^{32}P]dATP were from Dupont, NEN Research Products (Boston, Mass.). Isopropyl-β-D-thiogalactopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, RNase A, ampicillin, kanamycin, and carbenicillin were from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of analytical reagent grade.

Enzyme assays. The assay for the E1 component of branched-chain keto acid dehydrogenase was performed in

TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant genotype or phenotype[a]	Source or reference
P. putida		
PpG2	Wild type	I. C. Gunsalus
JS113	*bkdA1 bkdA2*	29
KT2440	*mt-2 hsdR1* (r^- m^+)	16
rpoN mutant	Km^r *rpoN*	16
E. coli		
TB1	*ara lacZ*ΔM15 Δ(*lac-proAB*) ϕ80 *hsdR17* (r^- m^+) *strA*	Bethesda Research Laboratories
DH5α	F^- ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 hsdR17* (r^- m^+) *recA1 supE44* lambda$^-$ *thi-1 gyrA relA1*	Bethesda Research Laboratories
JM101	Δ(*lac-proAB*) *supE thi*(F′ *traD36 proAB lacI*^{q}*Z*ΔM15)	31
Plasmids		
pKT240	IncQ *mob*$^+$ Ap^r Km^r	2
pJRS25	*bkd* promoter in pUC19, same orientation as *lacZ*	This study
pJRS40	*bkd* promoter in pUC19, opposite orientation as *lacZ*	This study
pJRS43	*bkd* promoter with *bkdA1* and *bkdA2* in pUC19, opposite orientation as *lacZ*	This study
pJRS44	*bkd* promoter with *bkdA* in pUC19, same orientation as *lacZ*	This study
pJRS47	Same insert as pJRS25 and pJRS40 in pKT240, opposite orientation as *aph*	This study
pJRS48	Same insert as pJRS25 and pJRS40 in pKT240, same orientation as *aph*	This study
pJRS49	Same insert as pJRS43 and pJRS44 in pKT240, opposite orientation as *aph*	This study
pJRS50	Same insert as pJRS43 and pJRS44 in pKT240, same orientation as *aph*	This study
pRK2013	ColE1 *mob*$^+$ *tra*$^+$ (RK2) Km^r	12
pUC19	Ap^r	31
Phage (M13mp19)		31

[a] Gene designations for *P. putida* are *bkdA1*, E1α subunit, and *bkdA2*, E1β subunit of branched-chain keto acid dehydrogenase.

the presence of excess E2 and LPD-Val. The latter two components were provided by a 90,000 × *g* supernatant fraction of *Escherichia coli* TB1(pJRS3) (28). The conditions of the assay for branched-chain keto acid dehydrogenase were described in reference 27. The assay for E1 activity used the same conditions except that the assay was supplemented with 300 μg of a 90,000 × *g* supernatant fraction prepared from *E. coli* TB1(pJRS3). This fraction supplies excess E2 and LPD-Val so that the rate of NADH formation depends on the amount of E1α and E1β.

Nucleic acid preparations. Plasmid and phage DNAs were prepared by the method described in reference 19, and RNA was prepared as reported earlier (6). Nick translation of DNA was performed according to the recommendations of the manufacturer with a kit from Bethesda Research Laboratories. Synthetic oligonucleotides were end labeled by the method of Maniatis et al. (19).

Screening of *P. putida* genomic library. An *Sph*I genomic library of *P. putida* DNA in pUC19 in *E. coli* TB1 was used. The nick-translated probe for screening the library was prepared from pSS1-2 (4) by digestion with *Sst*I and *Pst*I. This released a 1.45-kb fragment of DNA that included *bkdA1* and part of *bkdA2*. The library was plated on L agar containing ampicillin, and the colonies were lifted with a Colony/Plaque screen (Dupont, NEN Research Products). DNA fixation, hybridization, and washing conditions were those suggested by the manufacturer.

Subcloning and DNA sequencing. The genome DNA insert from the positive clone, pJRS25, was excised from pUC19 by digesting the DNA with *Sph*I, and the excised fragment was cloned in both orientations into the *Sph*I site of M13mp19. These clones were digested at the *Kpn*I and *Bam*HI sites of the vector, treated with exonuclease III and S1 nuclease, and ligated, yielding a set of ordered deletions (13) for DNA sequencing, which was done with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio). The nucleotide sequences of both strands of DNA were determined, and all clones were overlapping. To avoid band compressions due to a high G+C content, dITP was used in place of dGTP as suggested by the manufacturer. Samples were electrophoresed in 7 M urea–6% acrylamide (acrylamide to bisacrylamide ratio, 19:1) gels in 89 mM Tris–89 mM boric acid–2.5 mM EDTA (pH 8.3).

S1 nuclease and reverse transcriptase mapping. A clone containing bases 1 to 1354 of the strand encoding the *bkd* operon in M13mp19 was used to prepare radiolabeled, single-stranded DNA (1) to identify the start of transcription. A 17-mer universal primer was annealed to single-stranded DNA of the M13mp19 subclones, and the complementary strand was synthesized with [α-^{32}P]dCTP, deoxynucleoside triphosphates, and Klenow polymerase. To minimize the amount of uncopied M13mp19 template, we included a fivefold molar excess of primer and cold nucleotides in the synthesis reaction (7). RNA (50 μg) extracted from *P. putida* grown in valine-isoleucine or glucose synthetic medium and labeled DNA (10,000 cpm) were mixed in 30 μl of hybridization buffer (0.4 M NaCl, 0.2 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid] [pH 6.5], 5 mM EDTA, 80% formamide) (8). The solution was heated for 10 min at 75°C and incubated at 40°C overnight for hybridization of the DNA probe with branched-chain keto acid dehydrogenase-specific mRNA. Unhybridized DNA was digested with 500 U of S1 nuclease in S1 buffer (0.25 M NaCl, 30 mM potassium acetate [pH 4.5], 1 mM $ZnSO_4$, 5% glycerol) at 40°C for 1 h. Nucleic acids were extracted with phenol and precipitated with ethanol, and the pellet was dissolved in deionized formamide and tracking dyes. The solution was heated to denature nucleic acids and then loaded on a sequencing gel along with dideoxy sequencing ladders for precise sizing.

Reverse transcriptase mapping was done as described previously (24). A synthetic oligonucleotide, 5′-CTGCTGC

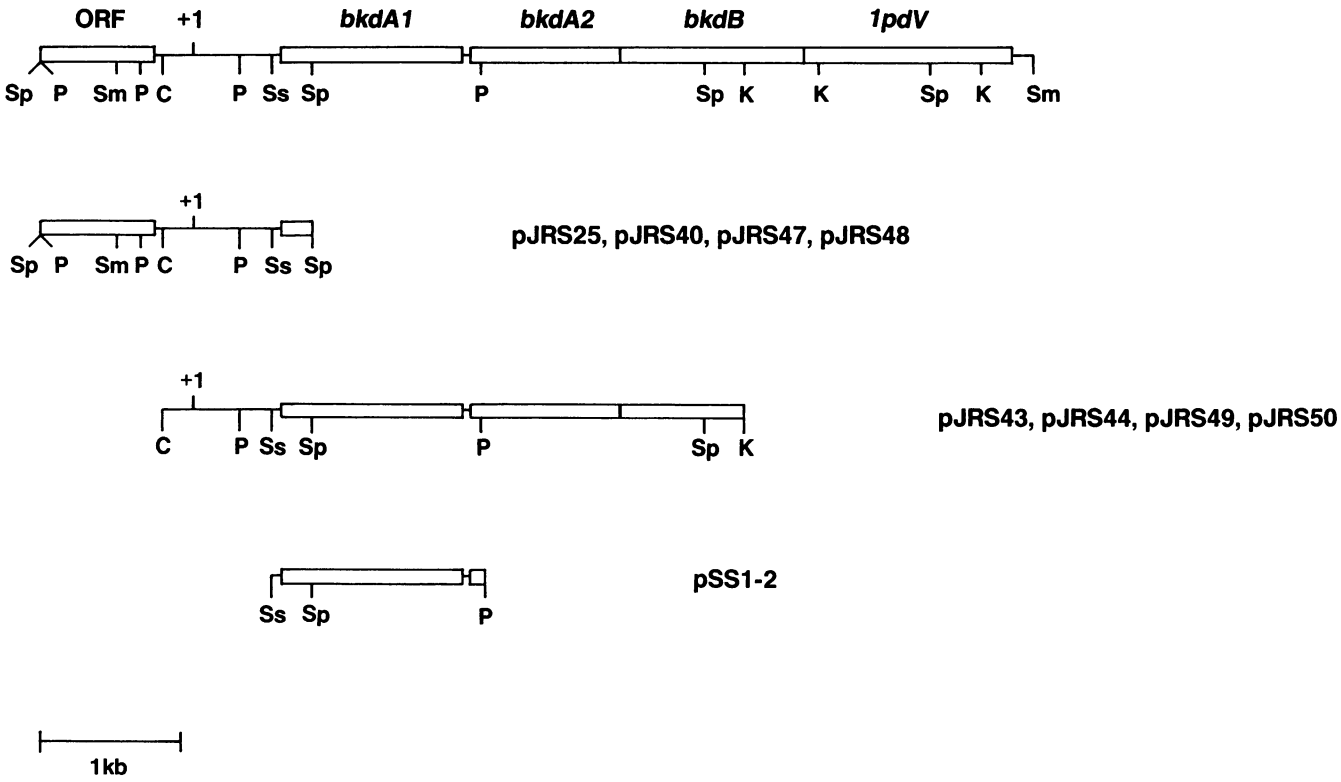

FIG. 1. Restriction map of the *bkd* operon and clones used in this study. The location of the transcriptional start of the operon is +1. The structural genes are as follows: *bkdA1*, E1α; *bkdA2*, E1β; *bkdB*, E2; and *lpdV*, LPD-Val. ORF indicates the unidentified open reading frame on the strand opposite that encoding the *bkd* operon. Abbreviations for the restriction enzyme sites are: C, *Cla*I; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I; Ss, *Sst*I; Sm, *Sma*I.

CGAGTATC-3′, beginning 546 base pairs (bp) upstream of the branched-chain keto acid dehydrogenase ATG initiation codon and complementary to the mRNA was used as a primer. It was synthesized at the Molecular Biology Resource Facility of the Saint Francis Hospital of Tulsa, Okla. The 5′-end-labeled primer (5,000 to 10,000 cpm) was combined with 50 μg of RNA from *P. putida* grown in valine-isoleucine medium. Samples were heated to 60°C for 15 min and cooled slowly to 42°C in 10 μl of buffer (50 mM tris [pH 8.3], 120 mM KCl). The remaining components of the reaction were added to a final volume of 50 μl in 50 mM tris (pH 8.3)–10 mM $MgCl_2$–135 mM KCl–5 mM dithiothreitol–0.7 mM deoxynucleotide triphosphates. After the addition of 15 U of avian myeloblastosis virus reverse transcriptase, samples were incubated for 1 h at 42°C and the reaction was stopped by bringing the temperature to 75°C for 10 min. After the reaction mixture cooled to 40°C, 5 μg of boiled RNase A was added to this mixture and further incubated for 1 h at 37°C. The nucleic acids were precipitated with ethanol and analyzed by electrophoresis as described above for S1 protection analysis.

Molecular cloning. The insert containing the *bkd* promoter was excised from pJRS25 by digestion with *Sph*I and inserted into pUC19. Two constructs were obtained, one with the insert in the same orientation as *lacZ*, that is, the same as pJRS25, and a second, pJRS40, which had the insert in the opposite orientation as *lacZ*.

To determine how much of the insert was required for promoter activity, we prepared a set of ordered deletions from these clones by digestion with exonuclease III and S1 nuclease (13). The inserts were excised from the multiple cloning sites of pUC19 by digestion with *Eco*RI and *Hin*dIII, isolated by agarose gel electrophoresis, and inserted into pKT240, also digested with *Eco*RI and *Hin*dIII. *E. coli* DH5α was the host for transformation, and transformants were selected by using L agar containing ampicillin. These constructs were transferred from *E. coli* DH5α to *P. putida* PpG2 by triparental mating (12), and the exconjugants were plated on pseudomonas isolation agar containing carbenicillin.

Clones with *bkdA1* and *bkdA2* as the reporter genes for the *bkd* promoter were constructed from pSO2, an 18-kb cosmid clone in pLAFR1 which contains the entire *bkd* operon plus 12 kb of flanking sequence (S. K. Oh, M.S. thesis, University of Oklahoma Health Sciences Center, Oklahoma City, 1989). The cosmid pSO2 was digested with *Sma*I, releasing a 6.8-kb fragment containing the entire operon which was inserted into the *Sma*I site of pUC19, yielding pJRS51. The insert was removed from pJRS51 by digestion with *Cla*I and *Bam*HI. The *Cla*I site is at base 828 (Fig. 1), and the *Bam*HI site is in the polylinker of pUC19. The ends of the insert were blunted with Klenow fragment and deoxynucleoside triphosphates, and the fragment was cloned into the *Sma*I site of pUC19. The resulting plasmid was digested with *Kpn*I, which cuts into *bkdB*, leaving *bkdA1* and *bkdA2* intact, and into the polylinker upstream of the operon (Fig. 1), and cloned into the *Kpn*I site of pUC19. Two constructs were obtained, pJRS43 with the promoter, *bkdA1*, and *bkdA2* in the opposite orientation as *lacZ*, and pPJRS44, with the insert in the same orientation as *lacZ*. The inserts were then isolated from pUC19 by digestion with *Eco*RI and *Hin*dIII and inserted into pKT240 similarly digested. Again,

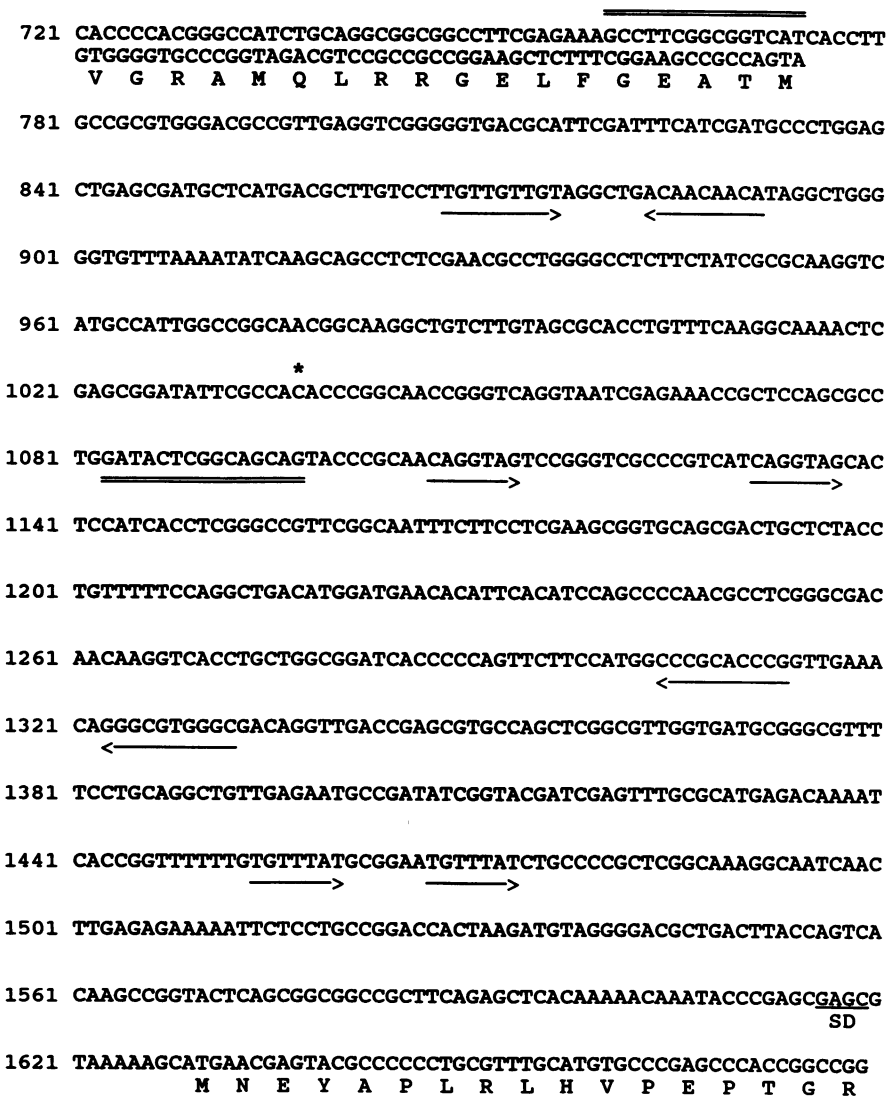

FIG. 2. Nucleotide sequence of the *bkd* leader. The translational start of *bkdA1* begins at 1629 bp and the unidentified open reading frame begins at base 774 on the strand opposite that of *bkd* genes. The transcriptional start of the *bkd* operon is indicated by an asterisk. Dyad and tandem repeats in the sequence are underlined with an arrow indicating the direction of the repeat. SD is the presumed Shine-Dalgarno ribosome-binding site. Double rules (═══) show regions where synthetic primers were used to identify transcriptional starts.

two constructs were obtained, pJRS49, which has the insert in the opposite orientation as the *aph* gene, and pJRS50, which has the insert in the same orientation as *aph*. The constructs were then transferred from *E. coli* DH5α to *P. putida* JS113 by triparental mating with pRK2013 (12).

Nucleotide sequence accession number. The GenBank accession number for the *bkd* promoter is M33715.

RESULTS

Isolation of *bkd* promoter. An *Sph*I genomic library of *P. putida* DNA in pUC19 was screened with a 1.45-kb nick-translated *Sst*I-*Pst*I fragment of *P. putida* DNA from pSS1-2 which contained all of *bkdA1* and part of *bkdA2* (Fig. 1) (4). Several positive colonies were identified during the initial screening which were further screened by restriction digestion of minipreparations and Southern blotting with the 1.45-kb probe. A clone containing a 1.87-kb insert was obtained that contained 244 bp of the *bkdA1* gene and 1,628 bp of upstream DNA. This clone was named pJRS25, and the restriction map of the insert is shown in Fig. 1.

Nucleotide sequence of pJRS25 insert. The nucleotide sequence of bases 721 to 1679 of pJRS25 is shown in Fig. 2. The codon for the initiating methionine of *bkdA1* starts at position 1629, and the translated amino acid sequence matched exactly that of E1α (4). The nucleotide sequence of the strand containing *bkdA1* was translated in all three frames, but no additional open reading frames were found on that strand, which means that there is a large noncoding segment of DNA upstream of *bkdA1* (Fig. 2). There is a region of dyad symmetry from bases 868 to 876 and 883 to 891 with a modest but probably significant free energy of formation of −14 kcal. The sequence beginning at base 1107, CAGGTAG, is repeated beginning at base 1131, and there is another tandem repeat at positions 1454 and 1467. There is a curious kind of symmetry beginning at position 1304, where the sequence CCCGCACCCG is followed by its comple-

ment, GGGCGTGGGC, beginning at position 1323. The G+C content of the leader sequence (bases 775 to 1628, Fig. 2) is 56.7%, which is distinctly lower than the 65.2% for the structural genes of the *bkd* operon (4–6). This agrees with the belief that RNA polymerase binds preferentially to A+T-rich regions of the promoter (30). A low G+C content of the promoter region might also contribute to promoter strength by providing less resistance to DNA unwinding. Similar observations of low G+C content of *Pseudomonas* promoters were made in the *algD* (9) and *nah* and *sal* (23) promoters.

The strand opposite that encoding the branched-chain keto acid dehydrogenase operon was translated into three reading frames, and an open reading frame was found starting at 774 bp (Fig. 2). However, there does not seem to be a strong ribosome-binding site preceding the start codon. This reading frame encoded 258 amino acids without a stop codon, and the codon usage was consistent with that of other *Pseudomonas* genes. The amino acid sequence was compared with the amino acid sequences of known regulatory proteins of bacteria in the Protein Information Resource data base, but no significant homology was found. However, a search by FASTP (18) showed modest homology with several glutamine synthetases in the literature. The values ranged from 22 to 31% identity over a span of about 130 amino acids and always to the same part of glutamine synthetase, residues 175 to 305.

Transcriptional start of *bkd* operon. The approximate start of the *bkd* transcript was first determined by S1 nuclease protection experiments. A single-stranded DNA template in M13mp19 was constructed by exonuclease III digestion (13) which included bases 1 to 1354 of the pJRS25 insert. The M13 sequencing primer was annealed to the single-stranded DNA, and the complementary strand was synthesized by using Klenow polymerase, deoxynucleoside triphosphates, and [α-^{32}P]dCTP. The radioactive DNA probe was hybridized to total cellular RNA extracted from *P. putida* grown in valine-isoleucine medium followed by treatment with a single-strand-specific S1 nuclease to destroy unprotected probe sequences (1, 7). These experiments indicated that the transcriptional start of the *bkd* operon was located about 600 bases upstream from the translational start (data not shown). To locate the transcriptional start precisely, we did reverse transcriptase mapping by primer extension. A 15-mer oligonucleotide was constructed complementary to bases 1083 to 1097, i.e., 59 bp downstream from the start of transcription (Fig. 2). The end-labeled primer was hybridized to cellular RNA from *P. putida* PpG2 grown on valine-isoleucine medium and extended the length of branched-chain keto acid dehydrogenase mRNA with avian myeloblastosis virus reverse transcriptase. The product was electrophoresed beside dideoxy sequencing reaction mixtures with the same oligonucleotide primer (Fig. 3). A single transcript was obtained, the mobility of which corresponded to base number 1037 of the pJRS25 insert. Therefore, the first base of the transcript is a cytidine nucleotide, which means that the distance between the transcriptional and translational starts is 592 bp.

To find the transcriptional initiation site of the message for the unknown open reading frame on the opposite strand, we did reverse transcriptase mapping using an 18-mer oligonucleotide that hybridized between bp 1097 and 1115 on the opposite strand (Fig. 2). The end-labeled oligonucleotide was annealed to RNA extracted from *P. putida* grown on L broth and minimal medium containing glucose or valine-isoleucine as the carbon source. No primer extension was evidenced after denaturing gel electrophoresis, indicating

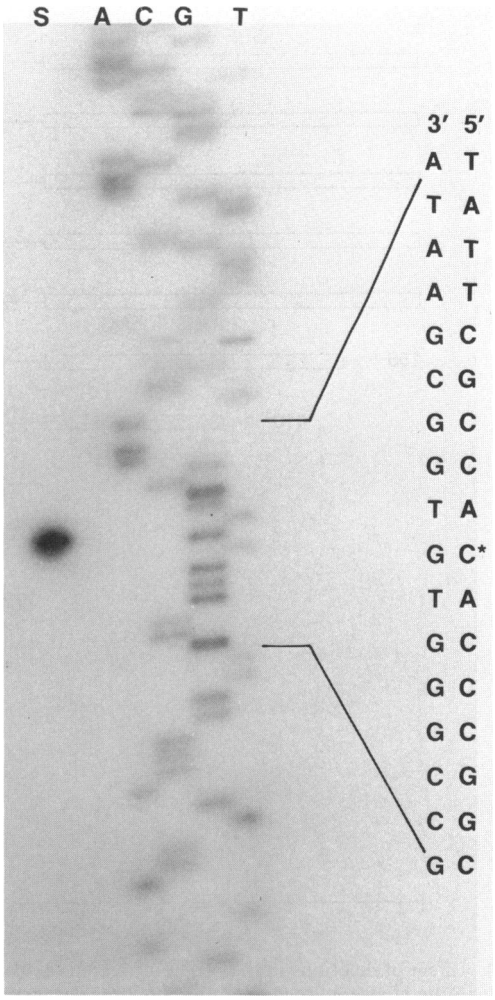

FIG. 3. Reverse transcriptase mapping of branched-chain keto acid dehydrogenase promoter. RNA (50 μg) isolated from *P. putida* grown in minimal medium containing 0.3% valine and 0.1% isoleucine was hybridized to a 15-bp primer complementary to the coding strand beginning 59 bp downstream from the start of transcription. Avian myeloblastosis virus reverse transcriptase was used to extend the primer the length of the mRNA. Lanes A, C, G, and T are dideoxy sequencing reactions with the 15-bp primer, and lane S is the primer extension product. The sequence in the region of the start of transcription is shown at the right, and the +1 nucleotide on the *bkd* coding strand is indicated by an asterisk.

that there may not be a transcript or that the transcript was not produced under the conditions in which the cells were grown. Thus, it is not clear whether we are dealing with two promoters or whether the *bkd* promoter is bidirectional.

Expression from the *bkd* promoter. The promoter activity of the insert of *P. putida* DNA in pJRS25 was studied with pKT240, which has a promoterless aminoglycoside phosphotransferase (*aph*) gene (2). When a DNA fragment containing a promoter is cloned in the correct orientation upstream of the *aph* gene, the host cell becomes streptomycin resistant. The entire insert of pJRS25 was cloned into pKT240 in both orientations, yielding pJRS47, with the insert opposed to *aph*, and pJRS48, with the insert in the same orientation as *aph* (Table 1). *E. coli* DH5α containing either pJRS47 or pJRS48 did not grow on L agar containing streptomycin at concentrations of 0.3 to 0.5 mg/ml, indicating that *E. coli* does not read the *bkd* promoter well.

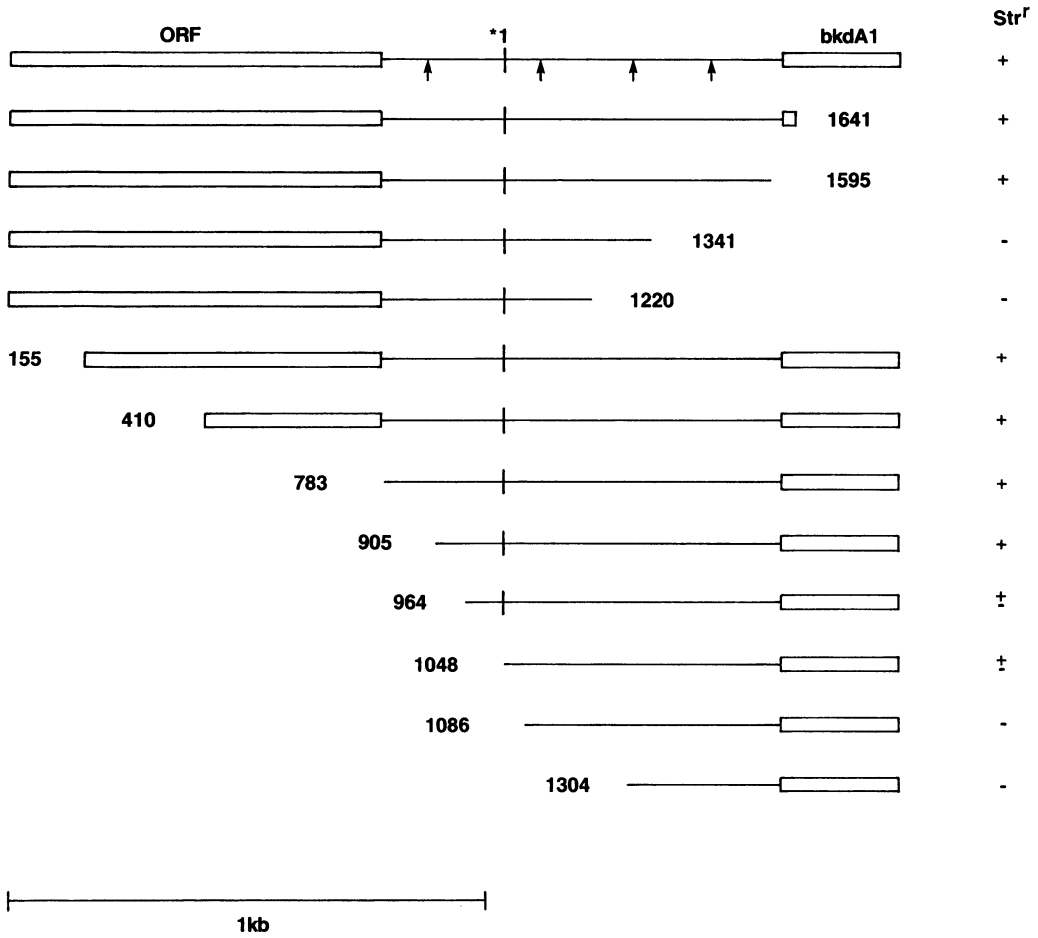

FIG. 4. Effect of deletions in the promoter region on *bkd* promoter activity. Streptomycin resistance (Str^r) was determined by growth on L agar plus 8 mg of streptomycin per ml. Numbers designate either the first (left) or last (right) base of the deletion clone as numbered in Fig. 2. ORF, Open reading frame.

pJRS47 and pJRS48 were then mobilized from *E. coli* DH5α to *P. putida* PpG2 by triparental mating (12), and the exconjugants were replica plated on minimal medium containing valine-isoleucine or glucose as the carbon source plus various concentrations of streptomycin. *P. putida* PpG2 containing either pJRS47 or pJRS48 was resistant to streptomycin at concentrations up to 10 mg/ml in both enriched and minimal media with either glucose or valine-isoleucine as the carbon source. *E. coli* DH5α(pKT240) and *P. putida* PpG2(pKT240) did not grow at streptomycin concentrations beyond 0.25 and 2 mg/ml, respectively. Expression of streptomycin resistance from pJRS47 was not expected, and this result suggests the presence of another promoter, possibly for the expression of the unidentified open reading frame on the strand opposite that of the *bkd* operon, or that the *bkd* promoter is bidirectional.

A series of ordered deletions were created in which the insert isolated from pJRS25 was shortened from both ends and then introduced into pKT240 (see Materials and Methods) to determine what effect this had on promoter activity (Fig. 4). There is a span of about 550 bp which is essential for promoter activity in *P. putida* from −100 bp upstream of the start of transcription to 450 bp downstream from the start of transcription. It is interesting that the two tandem repeats and the one dyad repeat downstream of the start are included in this essential region. However, the dyad repeat about 200 bp upstream of the start of transcription is not included. Perhaps this latter structure is involved in the expression of the unidentified open reading frame.

Expression of the *bkd* operon does not require the *rpoN* gene product. Recently, Köhler et al. (16) reported the construction of an *rpoN* mutant of *P. putida* KT2440. The *rpoN* mutant lost several metabolic functions including the ability to use branched-chain amino acids as carbon sources. To test the hypothesis that expression of the *bkd* operon might require RpoN, we mobilized four of the deletion clones into *P. putida* KT2440 and into the *rpoN* mutant. Two of the clones, those beginning at bases 783 and 905 (Fig. 4), conferred streptomycin resistance to both *P. putida* KT2440 and its *rpoN* mutant. The other two clones, those beginning at bases 1086 and 1304 (Fig. 4), failed to confer streptomycin resistance to either strain of *P. putida* KT2440. In addition, the *rpoN* mutant of *P. putida* KT2440 is able to grow in synthetic medium with 2-ketoisovalerate as the sole carbon source, so it is clear that the *rpoN* sigma factor is not required for expression of branched-chain keto acid dehydrogenase. As a control, we confirmed that the *rpoN* mutant cannot grow in medium with valine-isoleucine as the carbon source; hence, RpoN is required for either transport or transamination of branched-chain amino acids.

Expression of *bkdA1* and *bkdA2* from the *bkd* promoter. To study the expression of the *bkd* operon, we constructed

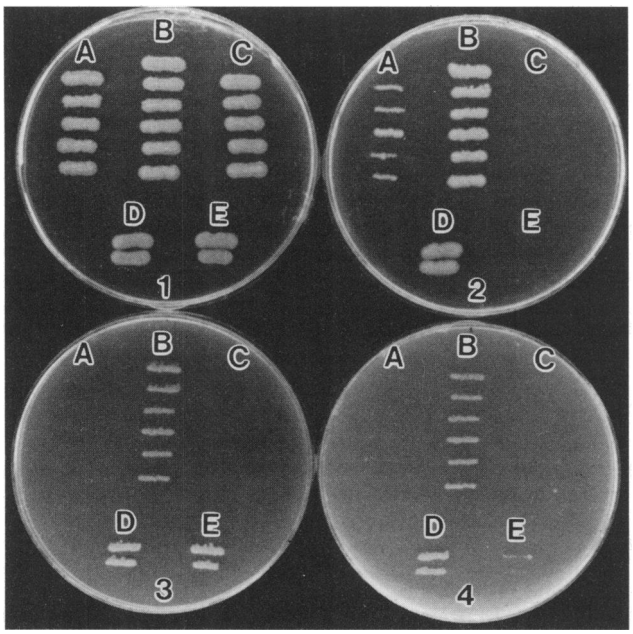

FIG. 5. Streptomycin resistance of *P. putida* JS113 containing pJRS49 and pJRS50. Plate 1, L agar; plate 2, L agar plus 8 mg of streptomycin per ml; plate 3, valine-isoleucine medium plus 2 mg of carbenicillin per ml; plate 4, valine-isoleucine medium plus 8 mg of streptomycin per ml. The cultures are *P. putida* JS113(pJRS49) (A), *P. putida* JS113(pJRS50) (B), *P. putida* JS113(pKT240) (C), *P. putida* PpG2(pJRS48) (D), and *P. putida* PpG2(pKT240) (E).

pJRS43 and pJRS44 (Fig. 1), which contain the *bkd* promoter, *bkdA1*, *bkdA2*, and part of *bkdB* in both orientations with respect to *lacZ* of pUC19 (Table 1) (see Materials and Methods). The insert from the pUC19 clones was then cloned into pKT240 in both orientations, yielding pJRS49, with the insert opposed to *aph*, and pJRS50, with the insert in the same orientation as *aph*. pJRS49 and pJRS50 were transferred to *P. putida* JS113, a *bkdA* mutant, by triparental mating and plated on several media (Fig. 5). *P. putida* JS113 containing either pJRS49 or pJRS50 grew on L agar plus 8 mg of streptomycin per ml; however, *P. putida* JS113 (pJRS50) was more resistant to streptomycin than *P. putida* carrying pJRS49 (Fig. 5). These results indicated that readthrough to *aph* occurred in both orientations. *P. putida* JS113(pJRS50) grew on valine-isoleucine agar plus streptomycin. Therefore, the insert complemented the mutation in *P. putida* JS113. However, *P. putida* JS113(pJRS49) did not grow on valine-isoleucine agar containing either carbenicillin or streptomycin (Fig. 5) for reasons which are unclear but may be related to interference by these antibiotics with expression of the cloned *bkd* genes.

To measure the level of expression of *bkdA1* and *bkdA2* from the *bkd* promoter, we performed E1 enzyme assays on 90,000 × *g* supernatant fractions prepared from cultures grown in the media shown in Table 2. There was very little expression of *bkdA1* and *bkdA2* in *E. coli* DH5α containing either pJRS49 or pJRS50. There was also low expression of E1 in *P. putida* JS113 containing either pJRS49 or pJRS50 grown in GAS medium. This is about what is obtained in wild-type *P. putida* PpG2 grown in valine-isoleucine medium. There was very high expression of E1 from either pJRS49 or pJRS50 in *P. putida* JS113 grown in GASV medium, about 50 to 100 times that obtained in *P. putida* PpG2 grown in the same medium (29). The expression of E1

TABLE 2. Expression of structural genes for E1 subunits of *P. putida* branched-chain keto acid dehydrogenase

Organism and medium[a]	Sp act[b] with plasmid:		
	pKT240	pJRS49	pJRS50
E. coli DH5α (GASV)	0	0.018	0.014
P. putida JS113			
L broth	0.009	2.32	2.83
GAS	0.004	0.10	0.11
GASV	0.023	1.74	5.91

[a] Compositions of media are given in Materials and Methods.
[b] Specific activity is micromoles of NADH per minute per milligram of protein. The assay is described in Materials and Methods.

from pJRS49 was always significantly lower than that obtained from pJRS50. These results indicate that the expression of the *bkd* operon in *P. putida* JS113 is inducible. There was very good expression of the *bkd* operon from either construct when *P. putida* JS113 was grown in L broth (Table 2). This was somewhat surprising since only borderline expression of the *bkd* operon was obtained in strain PpG2 grown in L broth.

DISCUSSION

There are several interesting features about the *bkd* promoter and the expression of the *bkd* operon. The long leader, all of which is required for expression (Fig. 4), and the potential for secondary structure in the leader suggest that regulation of expression of the *bkd* operon is complex. Earlier, we had shown that glucose repressed *bkd* gene expression by about 50%, whereas glucose plus ammonium ion repressed *bkd* gene expression by about 80% (28). These earlier findings plus the present results suggest the occurrence of both induction by branched-chain keto acids (20) and repression by glucose and ammonium ion, which agrees with the idea of complex regulation of *bkd* operon expression. The level of cyclic AMP in *P. putida* and *P. aeruginosa* does not fluctuate as a function of the carbon source (22), and therefore cyclic AMP is probably not involved in catabolite repression in these organisms.

Another reason for suspecting complex regulation of the *bkd* operon is the very long leader, which, as far as we are aware, is the longest promoter leader in a pseudomonad and one of the longest found in a procaryote. The next longest in a pseudomonad is the 367-bp leader of the *algD* promoter of *P. aeruginosa* (9). There is a leader of 1,153 bp in *Bacillus stearothermophilus* preceding the initiating methionine codon of the glyceraldehyde-3-phosphate dehydrogenase structural gene (3). The results presented in Fig. 4 indicate that all of the *bkd* leader is required for expression. There is also the possibility of secondary structure in the leader (Fig. 2 and 4) which could affect stability of the message. Thus, it seems unlikely that the only function of the leader is to act as a space between the promoter and the start of translation.

Finally, since the *bkd* operon is translated from a single message (6), there is the possibility of differential expression of the four genes. Curiously, the only significant intergenic space is between *bkdA1* and *bkdA2*, the genes encoding the E1α and E1β subunits, where there are 40 bp (4), and only two intergenic bases occur in the remainder of the operon. This suggests that there is translational coupling between the expression of these two genes.

An examination of the promoters of several *Pseudomonas* genes (8, 10, 14, 15, 32) failed to reveal any striking similarity

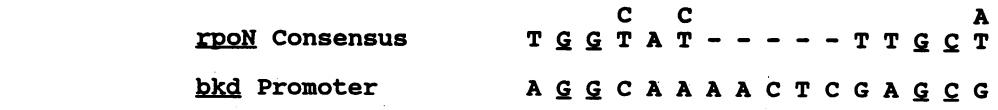

FIG. 6. Comparison of the *bkd* promoter region with that of the *rpoN* promoter (11). The underlined bases are the −12 and −24 consensus bases.

to the *bkd* promoter. The region upstream of the transcriptional start bears some resemblance to promoters of genes using the *rpoN* sigma factor (11), even though the experiments with the *rpoN* mutant of *P. putida* KT2440 showed that this sigma factor is not involved in *bkd* operon expression. There is a GC at −13 and −14, then 10 bases, and then a GG at −25 and −26 upstream of the transcriptional start, which is typical of *rpoN* promoters (Fig. 6). The fact that the operon is expressed so poorly in *E. coli*, giving about 0.2% of the activity obtained in *P. putida* JS113, indicates that *E. coli* does not read the *bkd* promoter well and/or does not contain the correct accessory proteins for *bkd* gene expression. Since the *rpoN* mutant of *P. putida* KT2440 grows with 2-ketoisovalerate as the sole carbon source, the *rpoN* gene product must be required for either transport of branched-chain amino acids or transamination.

ACKNOWLEDGMENT

This research was supported by Public Health Service grant DK 21737 from the National Institutes of Health.

LITERATURE CITED

1. **Aldea, M., F. Claverie-Martin, M. R. Diaz-Torres, and S. R. Kushner.** 1988. Transcript mapping using [^{35}S]DNA probes, trichloroacetate solvent and dideoxy sequencing ladders: a rapid method for identification of transcriptional start points. Gene **65**:101–110.
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. **Branlant, C., T. Oster, and G. Branlant.** 1989. Nucleotide sequence determination of the DNA region coding for *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase and of the flanking DNA regions required for its expression in *Escherichia coli*. Gene **75**:145–155.
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. **Calzone, F. J., R. J. Britten, and E. H. Davidson.** 1987. Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. Methods Enzymol. **152**:611–632.
8. **Debarbouille, M., and O. Raibaud.** 1983. Expression of the *Escherichia coli malPQ* operon remains unaffected after drastic alteration of its promoter. J. Bacteriol. **153**:1221–1227.
9. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15**:4567–4581.
10. **Deretic, V., W. M. Konyecsni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology **7**:1249–1254.
11. **Dixon, R.** 1986. The *xylABC* promoter from the *Pseudomonas putida* TOL plasmid is activated by nitrogen regulatory genes in *Escherichia coli*. Mol. Gen. Genet. **203**:129–136.
12. **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.
13. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
14. **Hu, L., S. L. Allison, and A. T. Phillips.** 1989. Identification of multiple repressor recognition sites in the *hut* system of *Pseudomonas putida*. J. Bacteriol. **171**:4189–4195.
15. **Koga, H., H. Aramaki, E. Yamaguchi, K. Takeuchi, T. Horiuchi, and I. C. Gunsalus.** 1986. *camR*, a negative regulator locus of the cytochrome P-450$_{cam}$ hydroxylase operon. J. Bacteriol. **166**:1089–1095.
16. **Köhler, T., S. Harayama, J.-L. Ramos, and K. N. Timmis.** 1989. Involvement of *Pseudomonas putida* RpoN sigma factor in regulation of various metabolic functions. J. Bacteriol. **171**: 4326–4333.
17. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–205.
18. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1987. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **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.
21. **McCully, V., G. Burns, and J. R. Sokatch.** 1986. Resolution of branched-chain oxo acid dehydrogenase complex of *Pseudomonas putida*. Biochem. J. **233**:737–742.
22. **Phillips, A. T., and L. M. Mulfinger.** 1981. Cyclic adenosine 3′,5′-monophosphate levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* during induction and carbon catabolite repression of histidase synthesis. J. Bacteriol. **145**:1286–1292.
23. **Schell, M.** 1986. Homology between nucleotide sequences of promoter regions of *nah* and *sal* operons of NAH7 plasmid of *Pseudomonas putida*. Proc. Natl. Acad. Sci. USA **83**:369–373.
24. **Shelness, G. S., and D. L. Williams.** 1984. Apolipoprotein II messenger RNA: transcriptional and splicing heterogeneity yields six 5′-untranslated leader sequences. J. Biol. Chem. **259**:9929–9935.
25. **Sokatch, J. R.** 1986. Metabolism of branched-chain amino acids in bacteria, p. 31–47. *In* R. Odessey (ed.), Problems and potential of branched-chain amino acids in physiology and medicine. Elsevier/North-Holland Publishing Co., Amsterdam.
26. **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.
27. **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.
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. **Vollenweider, H. J., M. Fiandt, and W. Szybalski.** 1979. A relationship between DNA helix stability and recognition sites for RNA polymerase. Science **205**:508–511.
31. **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.
32. **Zylstra, G. J., R. H. Olsen, and D. P. Ballou.** 1989. Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. **171**:5907–5914.