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Role of BkdR, a Transcriptional Activator of the SigL-Dependent Isoleucine and Valine Degradation Pathway in *Bacillus subtilis*

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A new gene, *bkdR* (formerly called *yqiR*), encoding a regulator with a central (catalytic) domain was found in *Bacillus subtilis*. This gene controls the utilization of isoleucine and valine as sole nitrogen sources. Seven genes, previously called *yqiS*, *yqiT*, *yqiU*, *yqiV*, *bfmBAA*, *bfmBAB*, and *bfmBB* and now referred to as *ptb*, *bcd*, *buk*, *lpd*, *bkdA1*, *bkdA2*, and *bkdB*, are located downstream from the *bkdR* gene in *B. subtilis*. The products of these genes are similar to phosphate butyryl coenzyme A transferase, leucine dehydrogenase, butyrate kinase, and four components of the branched-chain keto acid dehydrogenase complex: E3 (dihydrolipoamide dehydrogenase), E1α (dehydrogenase), E1β (decarboxylase), and E2 (dihydrolipoamide acyltransferase). Isoleucine and valine utilization was abolished in *bcd* and *bkdR* null mutants of *B. subtilis*. The seven genes appear to be organized as an operon, *bkd*, transcribed from a −12, −24 promoter. The expression of the *bkd* operon was induced by the presence of isoleucine or valine in the growth medium and depended upon the presence of the sigma factor SigL, a member of the sigma 54 family. Transcription of this operon was abolished in strains containing a null mutation in the regulatory gene *bkdR*. Deletion analysis showed that upstream activating sequences are involved in the expression of the *bkd* operon and are probably the target of *bkdR*. Transcription of the *bkd* operon is also negatively controlled by CodY, a global regulator of gene expression in response to nutritional conditions.

In *Bacillus subtilis*, the *sigL* gene encodes a sigma factor homologous to members of the RpoN family of sigma factors (5). Promoters recognized by an RNA polymerase associated with RpoN have common features: (i) they are devoid of typical −10 and −35 sequences but contain a strongly conserved TGGCAC N5 TTGCA sequence centered at positions −12 and −24, and (ii) they require a positive regulatory protein with a central domain (the catalytic domain) which includes a conserved nucleotide-binding pocket. This positive regulatory protein interacts with upstream activating sequences (UAS) to stimulate the isomerization of closed complexes between RNA polymerase and the promoter DNA sequences to open complexes (21). RpoN differs from other alternative sigma factors in that it is needed for the transcription of genes whose products have diverse physiological roles.

In *B. subtilis*, *sigL* mutants have a pleiotropic phenotype: transcription of the levanase operon is strongly reduced, and catabolism of several amino acids (arginine, ornithine, isoleucine, and valine) is abolished (5). Most studies of the catabolism of branched-chain amino acids have been done with *Pseudomonas*, *Streptomyces*, and *Streptococcus* species. In these bacteria, the catabolism of branched-chain amino acids requires the cooperation of two sequential series of reactions. The enzymes of the first series constitute a common pathway and catalyze the conversion by deamination or dehydrogenation of Leu, Val, and Ile to their respective 2-keto acids. Branched-chain 2-keto acid dehydrogenase, which catalyzes the second step in this initial process, is a multienzyme complex (BCDH complex) involved in the oxidation of the 2-keto acid derivatives of all three branched-chain amino acids. The acyl coenzyme A metabolites formed at the end of the common pathway are catabolized by a series of enzymes, one specific for each initial amino acid (19).

The BCDH complex from several sources has been characterized; these include *Pseudomonas aeruginosa* (20), *Pseudomonas putida* (32), *B. subtilis* (12), rabbit liver (25), and rat and bovine kidneys (24, 26). The purified complexes from *B. subtilis*, *P. putida*, *P. aeruginosa*, and several mammals are all composed of four polypeptides: a dehydrogenase (E1α), decarboxylase (E1β), a dihydrolipoamide acyltransferase (E2), and a dihydrolipoamide dehydrogenase (E3). In *B. subtilis*, the BCDH complex is involved in the biosynthesis of branched-chain fatty acids, which are the major acyl constituents of the cell membrane (37). A *bfmB* mutant of *B. subtilis* requiring short branched-chain carboxylic acids for growth has been described. It is defective in branched-chain 2-keto acid dehydrogenase. Three genes, *bfmBAA*, *bfmBAB*, and *bfmBB*, encode the E1α, E1β, and E2 components, respectively, of the BCDH complex involved in the biosynthesis of branched-chain fatty acids (35). Little is known concerning the regulation of the expression of the genes involved in isoleucine and valine catabolism in *B. subtilis*. A *sigL* mutant cannot use isoleucine or valine as a source of nitrogen, suggesting that the expression of one or several enzymes of the isoleucine and valine degradation pathway is controlled by a transcriptional regulator with a central domain (5).

In this paper, we characterize an operon containing seven genes involved in the isoleucine and valine degradation pathway. Transcription of this operon is induced by the presence of isoleucine or valine in the growth medium and strongly depends on SigL. We also describe a new transcriptional regulator, BkdR, which has a central domain and which is an activator of the transcription of this operon.

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or description[a]	Source or reference[b]
168	*trpC2*	1
QB5505	*trpC2 sigL*::*aphA3*	5
QB7501	*trpC2 amyE*::*ptb'-'lacZ* (ΔA)	This work
QB7502	*trpC2 amyE*::*ptb'-'lacZ sigL*::*aphA3*	QB5505 DNA→QB7501
QB7505	*trpC2 amyE*::*ptb'-'lacZ tnrA*::*erm*	SF706T DNA→QB7501
QB7507	*trpC2 lpd*::pMutin4	This work
QB7511	*trpC2 amyE*::*ptb'-'lacZ bkdR*::*aphA3*	pBkdR2 lin→QB7501
QB7512	*trpC2 bkdR*::*aphA3*	pBkdR2 lin→168
QB7513	*trpC2 lpd*::pMutin4 *bkdR*::*aphA3*	pBkdR2 lin→QB7507
QB7514	*trpC2 bcd*::pMutin4	This work
QB7515	*trpC2 bcd*::pMutin4 *bkdR*::*aphA3*	pBkdR2 lin→QB7514
QB7517	*trpC2 amyE*::*ptb'-'lacZ* (ΔB)	This work
QB7518	*trpC2 amyE*::*ptb'-'lacZ* (ΔC)	This work
QB7519	*trpC2 amyE*::*ptb'-'lacZ* (ΔD)	This work
QB7520	*trpC2 amyE*::*ptb'-'lacZ* (ΔE)	This work
QB7521	*trpC2 bkdB*::pMutin4	This work
QB7522	*trpC2 amyE*::*ptb'-'lacZ codY*::*erm*	PS258 DNA→QB7501
QB7530	*trpC2 amyE*::*ptb'-'lacZ bkdR*::*aphA3* pBkdR1	This work
PS258	*trpC2 codY*::*erm*	A. L. Sonenshein
SF706T	*trpC2 tnrA*::Tn*917 amyE*::*gabP-lacZ*	38

[a] ΔA, ΔB, ΔC, ΔD, and ΔE are defined in Fig. 5.
[b] lin, linearized. pBkdR1 and pBkdR2 were constructed as discussed in Materials and Methods.

TABLE 2. Doubling time of *bkd* mutants in TPK minimal medium containing ammonium, isoleucine, or valine as a nitrogen source

Strain	Relevant genotype	Doubling time (min) with indicated nitrogen source at 20 mM:		
		$(NH_4)_2SO_4$	Isoleucine	Valine
168		85	130	155
QB7512	*bkdR*::*aphA3*	150	>600	>600
QB5505	*sigL*::*aphA3*	70	>600	>600
QB7514				
Without IPTG	*bcd*::pMutin4	80	>600	>600
With IPTG		76	>600	>600
QB7507				
Without IPTG	*lpd*::pMutin4	75	170	200
With IPTG		115	160	190

MATERIALS AND METHODS

Bacterial strains and culture media. The *B. subtilis* strains used in this work are listed in Table 1. *Escherichia coli* TGI [K-12 Δ(*lac-pro*) *supE thi hsd5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15] was used for cloning experiments. *E. coli* was grown in LB broth (27), and *B. subtilis* was grown in SP medium (8 g of nutrient broth/liter, 1 mM $MgSO_4$, 10 mM KCl, 0.5 mM $CaCl_2$, 10 μM $MnCl_2$, 2 μM $FeSO_4$) or MM minimal medium [60 mM K_2HPO_4, 44 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 3 mM trisodium citrate, 2 mM $MgSO_4$, 2.2 mg of ferric ammonium citrate per liter] supplemented with carbon sources (0.1%) and auxotrophic requirements (at 100 mg/liter). TPK minimal medium contains 50 mM glucose, 100 mM potassium phosphate (pH 7.0), 0.5 mM $MgSO_4$, 0.01 mM $MnSO_4$, 0.02 mM FeCl, 50 μg of tryptophan per ml, and 20 mM nitrogen source.

Transformation and phenotype characterization. Standard procedures were used to transform *E. coli* (27), and transformants were selected on LB broth plates containing ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid or chromosomal DNA as previously described (16), and transformants were selected on SP medium plates containing chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), erythromycin (1 μg/ml), and lincomycin (10 μg/ml). Amylase activity in *B. subtilis* was detected after growth on tryptose blood agar base (Difco) containing 10 g of hydrolyzed starch per liter (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

DNA manipulation. Standard procedures were used to extract plasmid from *E. coli* (27). Restriction enzymes, phage T4 DNA polymerase, phage T4 DNA ligase, and T4 polynucleotide kinase were used as recommended by the manufacturers. DNA fragments were purified from the agarose gel with a Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.). The PCR technique with *Thermus aquaticus* DNA polymerase was used for amplification. The oligonucleotide primers used included mismatches allowing the creation of *Eco*RI and *Bam*HI restriction sites or *Hin*dIII and *Bam*HI restriction sites.

Plasmid constructions. pAC5 (17), a derivative of pAF1 (6), carries the pC194 chloramphenicol resistance gene *cat* and a *lacZ* gene between two fragments of the *B. subtilis amyE* gene. PCR was used to introduce *Eco*RI restriction sites at various positions upstream from *ptb*. PCR was performed with one oligonucleotide (5′-GGAGGATCCTCAGCATGAGCAAC-3′) corresponding to the coding sequence of the *ptb* gene (codons 20 to 25) and the other one corresponding to various positions in the *ptb* promoter region. The *Eco*RI-*Bam*HI restriction fragments generated in this way were inserted between the *Eco*RI and *Bam*HI restriction sites of pAC5, creating translational fusions between codon 25 of *ptb* and codon 8 of *lacZ*. The DNA sequences of the different PCR fragments were verified by direct sequencing of the various corresponding plasmids. The resulting plasmids were linearized at the single *Pst*I restriction site and integrated into the chromosome of strain 168 by homologous recombination at the *amyE* locus by use of chloramphenicol selection. Integrants carrying the translational fusions were named QB7501, QB7517, QB7518, QB7519, and QB7520 (see Fig. 5 and Table 1).

Gene disruptions and transcriptional fusions with the pMutin4 vector (34) were constructed by PCR amplification of an internal segment of the target gene, ligation of the amplified DNA fragment into pMutin4 in *E. coli*, and insertion of the plasmid into the *B. subtilis* chromosome. PCR amplifications were done with the following oligonucleotides: 5′-GCCGAAGCTTGGCCGCTATATTCAAG G-3′ and 5′-CGCGGATCCGGCTACGTTCCCAACAC-3′ for the *bcd* gene; 5′-GCCGAAGCTTCTGTTGAGCGGGGAAAT-3′ and 5′-CGCGGATCCGG CAGCACTTTTGCCCC-3′ for the *lpd* gene; and 5′-GCCGAAGCTTGACCTC GATCAAGTGAC-3′ and 5′-CGCGGATCCAATTTTGTCCCCCGCCC-3′ for the *bkdB* gene. Target genes were interrupted by Campbell-type crossover integrations. The integration of the recombinant plasmids places the downstream genes under the control of the *spac* promoter regulated by isopropyl-β-D-thiogalactopyranoside (IPTG) and fuses the target gene to the *lacZ* gene, leading to a transcriptional fusion.

pBkR1, which contains the wild-type *bkdR* regulatory gene, was constructed as follows. Two DNA fragments encoding the amino-terminal part and the carboxy-terminal part of BkdR were synthesized by PCR with the following respective pairs of oligonucleotides: (i) 5′-CCGGAATTCGTGGTAACATAGGGTTG-3′ and 5′-TCCCCCGGGTTCCCGGCATGACAATG-3′ and (ii) 5′-TCCCCCGG GTATCCGATCTCCATCC-3′ and 5′-CGCGGATCCGGGTGCTGTCATACC AGG-3′. The two DNA fragments were ligated into pHT315 (2) between the *Eco*RI and *Bam*HI restriction sites and on each side of a DNA fragment containing the *aphA3* gene (33), leading to pBkR2.

The interrupted gene was introduced into the chromosomes of strain 168 to give strain QB7512 and strain QB7501 to give strain QB7511. The wild-type *bkdR* gene was cloned by homologous recombination as follows. Strain 168 containing pBkR2 was grown in LB medium containing erythromycin. Plasmid DNA was extracted and used to transform strain QB7511 containing the *ptb'-'lacZ* fusion.

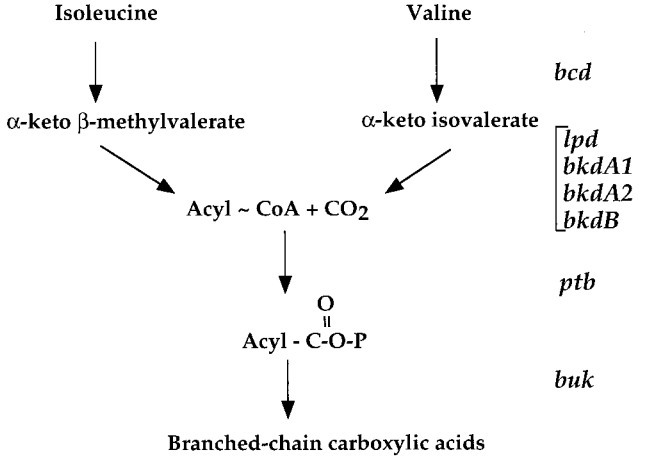

FIG. 1. Proposed pathway for the degradation of branched-chain amino acids in *B. subtilis*.

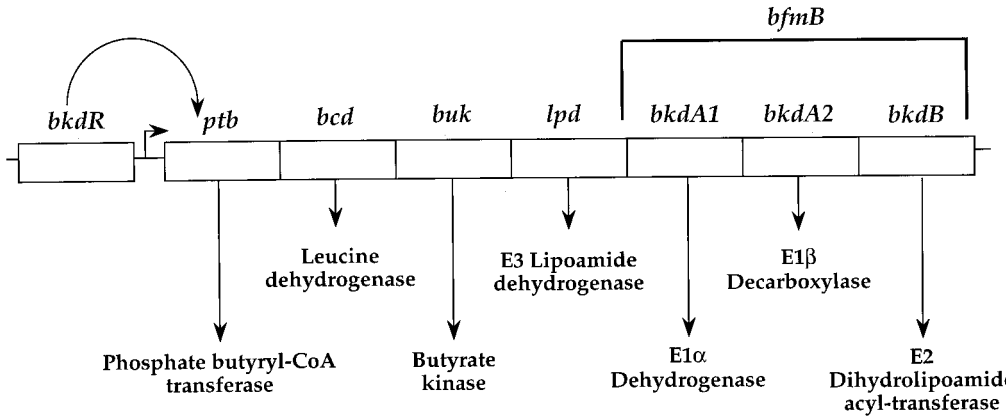

FIG. 2. Organization of the structural genes of the *bkd* operon of *B. subtilis*. The proposed functions of the gene products are based on similarities to the corresponding genes from *Enterococcus faecalis* (36). CoA, coenzyme A.

Transformants were selected on SP medium plates containing chloramphenicol and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). One blue colony was reisolated, and plasmid DNA (pBkR1) was extracted. This plasmid contains the entire *bkdR* gene.

Reverse transcriptase mapping of the mRNA start point in the *ptb* gene. Total RNA was isolated from *B. subtilis* 168 grown in MM medium supplemented with glucose and tryptophan and with or without 20 mM isoleucine as the inducer. Exponentially growing cells were harvested at an optical density at 600 nm of 1, and RNA was extracted as previously described (8). Two oligonucleotides, O_1 (5′-CCTCAGCATGAGCAACCGCAATGGTC-3′) and O_2 (5′-GTGTATCGA CGCTTTGCCGATTAAATC-3′), complementary to the *ptb* coding sequence were labelled with 10 U of polynucleotide kinase and 0.37 MBq of [γ-^{32}P]ATP (15 TBq/mmol; Amersham). DNA primers were elongated, and the products were analyzed as previously described (15) and shown to have the same transcriptional start sites.

β-Galactosidase assays. *B. subtilis* cells containing *lacZ* fusions were grown to an optical density at 600 nm of 1. β-Galactosidase specific activities were determined as previously described and are expressed as Miller units per milligram of protein (5). The values reported represent averages from at least three independent assays.

RESULTS

Identification of an activator involved in isoleucine utilization in *B. subtilis*. Members of the family of activators of σ^{54}-dependent transcription are composed of three distinct functional domains, an NH_2-terminal domain, a central domain, and a COOH-terminal domain, involved in signal reception, transcriptional activation, and DNA binding, respectively. The central domain and the C-terminal domain are the most highly conserved among these regulators (30). Degenerate oligonucleotide primers have been used to amplify DNA fragments encoding central domains from the genomes of diverse bacteria, including *B. subtilis*. This procedure led to the identification of both known genes (*levR* and *rocR*) and two novel gene fragments, called 70-Bsu and 81-Bsu (9). The 70-Bsu DNA fragment (a gift from I. Kaufman and T. Nixon) was cloned in pHT181, an integrative plasmid (11). The recombinant plasmid was used to transform *B. subtilis* 168. Since the 70-Bsu DNA fragment is an internal fragment of the gene, the integration led to a null mutation in the corresponding chromosomal gene. The *B. subtilis* mutant strain containing pHT181::70-Bsu inserted by homologous recombination in the corresponding gene was tested for growth in TPK minimal medium containing isoleucine (20 mM) as the sole nitrogen source. This strain grew much more slowly than the wild-type strain in the same medium containing isoleucine (data not shown). The mutant strain and the wild-type strain grew well with $(NH_4)_2SO_4$ as the sole nitrogen source. These results strongly suggest that the DNA fragment encoding a new central domain is part of a regulatory gene controlling isoleucine utilization in *B. subtilis*.

A new gene, *bkdR* (formerly *yqiR*), was found during the *B. subtilis* genome sequencing project (10). This gene encodes a protein with a central domain identical to the peptide deduced from the 70-Bsu fragment (9). The polypeptide deduced from the DNA sequence of *bkdR* contains 692 residues with a calculated molecular weight of 77,747. We constructed a *B. subtilis* strain in which *bkdR* was disrupted by the *aphA3* gene: this disruption was created by transformation and recombination into the chromosome of wild-type *B. subtilis* 168, leading to strain QB7512.

Strain QB7512 was tested for growth in TPK minimal medium containing the branched-chain amino acid isoleucine or valine as the sole nitrogen source (Table 2). It did not grow in the presence of isoleucine or valine as the sole nitrogen source, confirming that the product of the *bkdR* gene is involved in the control of the catabolism of these amino acids. *bkdR* and *sigL* null mutants can grow with $(NH_4)_2SO_4$ as the sole nitrogen source. However, the *bkdR* mutant grows slightly more slowly than the *sigL* mutant (see Discussion). The products of several genes located downstream from *bkdR* are presumably involved in the metabolism of branched-chain amino acids, since they show strong similarities to phosphate butyryltransferase, leucine dehydrogenase, butyrate kinase, and the E3, E1α, E1β, and E2 components of the BCDH complex (Fig. 1 and 2) (22). As shown below, these genes probably form an operon containing the following genes: *ptb*, *bcd*, *buk*, *lpd*, *bkdA1*, *bkdA2*, and *bkdB* (formerly *yqiS*, *yqiT*, *yqiU*, *yqiV*, *bfmBAA*, *bfmBAB*, and *bfmB*, respectively) (Fig. 2).

To test whether the transcription of these genes is induced by isoleucine or valine in the growth medium, a *ptb′-′lacZ* translational fusion was constructed and integrated at the *amyE* locus of *B. subtilis* 168, leading to strain QB7501. The level of β-galactosidase expression in this strain was assayed (Table 3), and the transcription of *ptb* was indeed induced by isoleucine or valine in the growth medium. Transcription was also induced, albeit to a lesser extent, when α-keto acids corresponding either to isoleucine (α-keto-β-methylvalerate) or to valine (α-ketoisovalerate) were present in the growth medium. A *sigL*::*aphA3* null mutation was introduced by transformation of fusion strain QB7501 to give strain QB7502. The expression of the *ptb′-′lacZ* translational fusion strongly depended upon the *sigL* gene product (Table 3). The product of the *bkdR* gene shows similarities to transcriptional activators required to stimulate −12, −24 promoters. A *bkdR*::*aphA3*

TABLE 3. Effects of *bkdR* and *sigL* mutations on *ptb'-'lacZ* expression

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein) in the following minimal medium[a]:				
		MM	MM + isoleucine (20 mM)	MM + α-keto-β-methylvalerate (20 mM)	MM + valine (20 mM)	MM + α-keto-isovalerate (20 mM)
QB7501	*amyE*::*ptb'-'lacZ*	55	2,265	950	1,145	400
QB7502	*amyE*::*ptb'-'lacZ sigL*::*aphA3*	5	5	ND	5	ND
QB7511	*amyE*::*ptb'-'lacZ bkdR*::*aphA3*	5	1	ND	5	ND
QB7530	*amyE*::*ptb'-'lacZ bkdR*::*aphA3* pBkdR1	140	2,440	ND	2,120	ND

[a] ND, not determined.

null mutation was introduced by transformation into the chromosome of strain QB7501, leading to strain QB7511. The β-galactosidase activity in cultures of this strain in MM glucose minimal medium containing 20 mM isoleucine or valine as the inducer was assayed. The expression of the *ptb'-'lacZ* fusion was extremely low in the absence of BkdR (Table 3). BkdR is therefore required for full induction of *ptb* transcription. pBkdR1, containing the entire coding sequence of the positive regulator, was introduced by transformation into strain QB7511, leading to strain QB7530. Transformants were grown in MM glucose minimal medium containing isoleucine or valine. The intact *bkdR* gene restored *ptb* transcription (Table 3), confirming that BkdR stimulates transcription via the synthesis of an activator protein.

Promoter and control regions located upstream from the *ptb* gene. In gram-negative and gram-positive bacteria, all promoters recognized by holoenzymes containing σ^{54} possess at least a conserved GG doublet located at position −24 upstream from the transcriptional start site, followed by a GC doublet at position −12, with a spacing region of 10 bp. They do not have the typical −10 and −35 hexamers recognized by the major housekeeping sigma factor (21). The transcriptional start site of *ptb* was mapped by primer extension with reverse transcriptase and two oligonucleotides, O_1 and O_2. The same 5′ ends were identified with each oligonucleotide (Fig. 3). Transcription started at two adjacent nucleotides. The promoter region contains a region consistent with the consensus sequence (−12 and −24) of σ^{54}-dependent promoters (Fig. 4).

σ^{54}-dependent promoters contain regulatory sequences, called UAS, which bind to specific activators. Many of the binding sequences are inverted repeats that can be moved away from their original positions without losing their ability to allow transcriptional activation (4, 13, 17). To determine if such sequences were necessary for the expression of the *ptb* gene, a set of DNA fragments from which parts of the upstream region were deleted were constructed by PCR. These fragments, which contain the beginning of the *ptb* gene and the promoter region, were cloned upstream from the *lacZ* gene (see Materials and Methods). Deletion end points are indicated in Fig. 5. Fusions were reintroduced as single copies at the *amyE* locus of *B. subtilis* 168. *lacZ* expression in the resulting strains in MM glucose minimal medium containing isoleucine was assayed (Fig. 5).

lacZ expression was higher for ΔB than for ΔC deletions, indicating that DNA sequences located between the ΔB and ΔC end points are necessary for full promoter activation. There was no *lacZ* expression in ΔD (or ΔE) strains. Thus, DNA sequences near position −107 (the ΔC end point) with respect to the transcriptional start site are necessary for full induction of the *ptb* gene. Interestingly, three copies of an imperfect palindromic DNA sequence (Fig. 5) are present upstream from the *ptb* −12, −24 promoter. Since deletion analysis showed a sharp decrease in the rate of β-galactosidase synthesis when these palindromes were absent, the product of *bkdR* may interact with these repeated sequences to stimulate transcription.

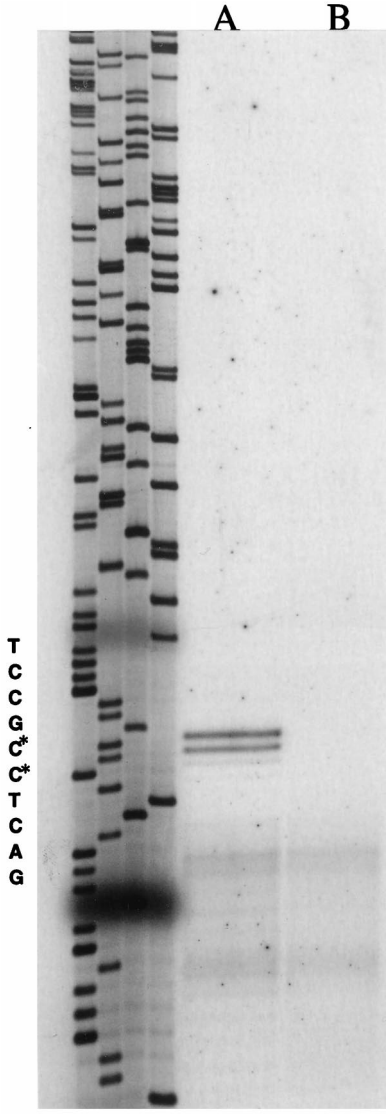

FIG. 3. Reverse transcriptase mapping of the transcriptional start site of the *ptb* gene in *B. subtilis* 168 grown in the presence (lane A) or absence (lane B) of 20 mM isoleucine. The positions of the cDNA-extended fragments identified with oligonucleotide O_1 were compared with those obtained by sequencing of an M13 recombinant phage containing the promoter region with the same oligonucleotide used as a primer (lanes at left, TCGA, respectively, from left to right). Transcriptional start sites are indicated by the asterisks.

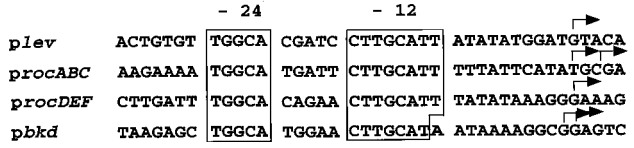

FIG. 4. Nucleotide sequences of promoter regions of the levanase operon (p*lev*), the *rocABC* operon (p*rocABC*), the *rocDEF* operon (p*rocDEF*), and the *bkd* operon (p*bkd*). The transcriptional start sites are indicated by arrows. Boxes indicate strictly conserved DNA sequences around positions −12 and −24 with respect to the transcriptional start sites.

Induction of the expression of the *bcd*, *lpd*, and *bkdB* genes. pMutin4 (34) was used to construct transcriptional fusions with the *lacZ* gene. These constructions were integrated by homologous recombination into the *bcd*, *lpd*, and *bkdB* genes, leading to strains QB7514, QB7507, and QB7521, respectively. The recombination event was mediated by DNA fragments generated by PCR, inserted into the vector, and corresponding to the internal part of each gene. Integration of the plasmids (i) inactivates the corresponding gene; (ii) fuses the target gene to the *lacZ* gene; and (iii) allows the expression of downstream genes from an IPTG-inducible promoter, *pspac*. The β-galactosidase activities of strains QB7514, QB7507, and QB7521 were assayed after culturing in MM glucose minimal medium containing or not containing 20 mM isoleucine as the inducer (Table 4). The β-galactosidase expression in strains QB7514, QB7507, and QB7521 was induced by 20 mM isoleucine in the growth medium. The uninduced level of β-galactosidase expression was about 300 U/mg of protein. The addition of 1 mM IPTG to the growth medium led to a strong decrease in *lacZ* expression from the *bcd′-lacZ* fusion, suggesting that the product of one or several genes downstream from *bcd* represses the −12, −24 promoter (see Discussion). A *bkdR*::*aphA3* null mutation was introduced into the *bcd′-lacZ* strain and into the *lpd′-lacZ* strain by transformation, leading to strains QB7515 and QB7513, respectively. β-Galactosidase was not expressed in cultures of these strains, whether or not they contained isoleucine as the inducer. Attempts to combine a *bkdB*::pMutin4 null mutation with either a *sigL* or a *bkdR* null mutation were unsuccessful, suggesting that three of the four subunits of the BCDH complex are essential for cell viability.

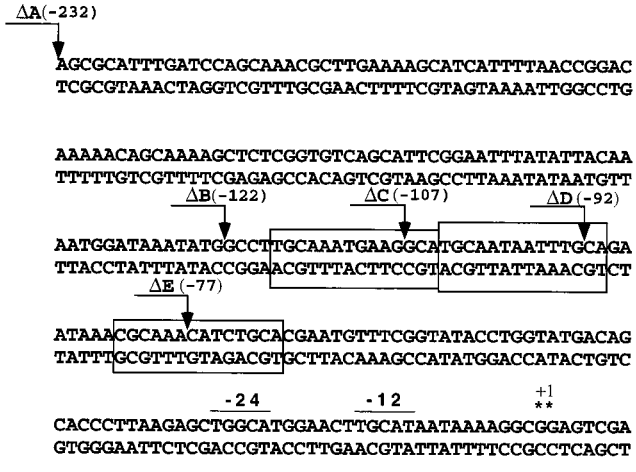

FIG. 5. Nucleotide sequence of the *ptb* upstream region. Deletion end points are indicated by arrows and numbered with respect to the transcriptional start site (indicated by asterisks). The sequences at positions −12 and −24 are indicated. Boxed regions indicate putative UAS. The effects of upstream deletions on the expression of the *ptb′-′lacZ* translational fusions are expressed as β-galactosidase specific activities, which were determined with extracts prepared from cells growing exponentially in MM minimal medium containing glucose and 20 mM isoleucine as the inducer. In the absence of isoleucine, the basal level was about 5 U/mg of protein for each strain. For deletion end points ΔA (−232), ΔB (−122), ΔC (−107), ΔD (−92), and ΔE (−77), β-galactosidase activities were 2,350, 2,135, 205, 5, and 10 U/mg of protein, respectively.

The results presented above indicated that the expression of the *ptb*, *bcd*, *lpd*, and *bkdB* genes is induced by isoleucine in the growth medium. Since transcriptional activation of *ptb*, *bcd*, and *lpd* also requires the product of *bkdR*, it is likely that these genes are cotranscribed as an operon.

Disruption of *bkdR* and phenotypes of *bcd*, *lpd*, and *bkdB* mutants. *B. subtilis* QB7512 (containing the *aphA3* gene inserted into *bkdR*) cannot grow in the presence of the branched-chain amino acids isoleucine and valine as the sole nitrogen sources. Thus, the product of the *bkdR* gene is involved in the control of the catabolism of these amino acids (Table 2). A similar phenotype was observed for strain QB7514, in which the pMutin4 vector was integrated into the *bcd* gene (Table 2). This strain was unable to use isoleucine or valine as a sole nitrogen source. Moreover, the doubling time with ammonia as a nitrogen source was unaffected. This result indicates that the *bcd* gene product, which shows similarities to leucine dehydrogenases, is involved in isoleucine and valine catabolism.

pMutin4 was also integrated into the *lpd* gene (strain QB7507). The integration of the plasmid prevents the production of a polypeptide showing similarities to the E3 component of the BCDH complex. As expected, the growth of this strain was unaffected when isoleucine or valine was used as a nitrogen source in the growth medium. Indeed, in this case, the functional *bcd* gene product led to the production of ammonia and α-keto acids. This result shows that the *lpd* gene is not essential in *B. subtilis*. However, two other lipoamide dehydrogenase homologs exist in *B. subtilis*, and we cannot exclude complementation of the *lpd* null mutant. Moreover, the *pspac* promoter is not tightly controlled by the product of *lacI*, leading to a low level of transcription of the downstream genes (34). Strain QB7521 (*bkdB*::pMutin4) grew slowly, and the cells lysed in both rich and minimal media, indicating that the product of *bkdB* is necessary for normal growth.

Regulation of the operon in response to nitrogen or amino acid availability. Two *B. subtilis* proteins, TnrA and CodY, are known to be involved in regulation by the nitrogen source and by an excess of amino acids, respectively. TnrA is a transcriptional factor required for global nitrogen regulation in *B. subtilis* (38). During nitrogen limitation, the TnrA protein is required for activation of the transcription of *gabP*, *nasB*, and *nrgAB* and negatively regulates *glnRA* expression. We introduced a *trnA*::*erm* mutation into strain QB7501 to give strain QB7505. *lacZ* expression in cultures of this strain during growth with the poor nitrogen source glutamate was assayed. In the absence of an inducer, *lacZ* expression in MN (7) glucose minimal medium containing glutamate was 4.5-fold higher in strain QB7505 than in strain QB7501. These results suggest that the *tnrA* gene product may act as a repressor of the operon. The presence of 20 mM isoleucine did not affect the induced level of expression in *tnrA* mutants (data not shown; see Discussion).

The expression of several genes and operons, including those for histidine degradation (*hut*), proline degradation, aconitase, and dipeptide permease (*dpp*), is repressed when the medium contains a mixture of amino acids. To assess the role of nutritional repression by amino acids, strain QB7501 was grown in MM glucose minimal medium with and without Casamino Acids (0.2% final concentration). The presence of Casamino Acids in MM glucose minimal medium containing

TABLE 4. Induction of the expression of *bcd*, *lpd*, and *bkdB* genes

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein) in the following minimal medium[a]:			
		MM + glucose		MM + glucose + isoleucine (20 mM)	
		Without IPTG	With IPTG	Without IPTG	With IPTG
QB7514	*bcd*::pMutin4	355	10	1,170	1,100
QB7515	*bcd*::pMutin4 *bkdR*::*aphA3*	2	2	3	2
QB7507	*lpd*::pMutin4	320	280	2,800	2,630
QB7513	*lpd*::pMutin4 *bkdR*::*aphA3*	5	5	3	4
QB7521	*bkdB*::pMutin4	290	ND	1,150	ND

[a] ND, not determined.

20 mM isoleucine as the inducer led to a 3.5-fold decrease in *lacZ* expression (Table 5). CodY is a global regulator of gene expression that mediates amino acid repression of the *hut* and *dpp* operons and the genetic competence pathway (28, 29, 31). We thus introduced a *codY*::*erm* mutation into strain QB7501 to give strain QB7522. Strain QB7522 was grown in MM minimal glucose medium containing isoleucine or Casamino Acids. The *codY*::*erm* mutation led to derepression of the *ptb'-'lacZ* fusion (Table 5). The mixture of amino acids did not repress expression in the *codY*::*erm* strain. The *codY* mutation appears, therefore, to relieve amino acid repression of the *bkd* operon, as previously observed for the *dpp* and *hut* operons. The *codY* mutation did not alter the expression of the fusion in MM glucose minimal medium containing isoleucine. However, partial constitutive expression of the *ptb'-'lacZ* fusion was observed in the absence of isoleucine.

DISCUSSION

The product of *sigL* is involved in the catabolism of isoleucine or valine present as the sole nitrogen source (5). In this work, we have identified an operon, the *bkd* operon, containing seven genes and involved in the deamination of isoleucine and valine and in the oxidative decarboxylation of branched-chain α-keto acids. Recently, a cluster of seven genes with a similar organization and involved in the breakdown of branched-chain amino acids was found in *Enterococcus faecalis*. Four of these genes, *ldp*, *bkdA1*, *bkdA2*, and *bkdB*, encode the E3, E1α, E1β, and E2 components of a BCDH complex. The genes preceding *lpd* in *E. faecalis*, *ptb* and *buk*, are similar to phosphotransbutyrylase and butyrate kinase genes, respectively, and are probably involved in ATP generation (36).

Here, we have shown that transcription of the *bkd* operon in *B. subtilis* is induced by the presence of isoleucine or valine in the growth medium. The α-keto acids α-keto-β-methylvalerate and α-ketoisovalerate are also inducers, a situation previously observed with *P. putida* (14). In a *bcd* null mutant (QB7514), the β-galactosidase expression observed in the absence of isoleucine in the growth medium was decreased by the presence of IPTG, which induces the expression of the downstream genes. As branched-chain amino acids or α-keto acids are probably internal inducers, it is likely that the intracellular levels of these inducers depend upon the expression of the downstream genes *lpd*, *bkdA1*, *bkdA2*, and *bkdB*. The *bfmB* mutant of *B. subtilis* requires branched short-chain carboxylic acids for growth. However, the *lpd* and *bkdB* genes could be interrupted without a loss of cell viability. Transcription of the operon is SigL dependent, and it was previously shown that mutants affected in the BCDH complex require branched-chain α-keto acids for growth. *sigL* and *bkdR* null mutants are viable, and it is therefore likely that the four genes encoding the different polypeptides of the BCDH complex are constitutively transcribed at a low level to ensure the synthesis of membrane branched-chain carboxylic acids. This basal level of transcription could be due to read-through transcription from the *bkdR* promoter upstream from the operon. The *bkdR* null mutant was constructed by inserting an antibiotic resistance cassette in *bkdR*. This cassette might affect the basal level of transcription of the operon, which could explain the slower rate of growth of the *bkdR* mutant than of the *sigL* mutant with ammonia as the sole nitrogen source. The expression of the operon is also subject to global regulation. During exponential growth in MM glucose minimal medium containing isoleucine, the expression of the *bkd* operon is slightly repressed by the addition of Casamino Acids, and this repression is *codY* dependent. The uninduced level of the *bkd* operon increases 3-fold in *tnrA* mutants, 7-fold in the *codY* mutant in MM minimal medium containing glucose, and 17-fold in the presence of Casamino Acids.

CodY is a repressor which binds to promoters in AT-rich regions, known to exhibit bending and curvature. Interestingly, looped DNA structures are involved in the formation of contacts between $E\sigma^{54}$ holoenzymes and enhancer binding proteins. However, it would be interesting to test whether TnrA and CodY bind directly to the *bkd* promoter region. Another possibility is that the intracellular level of the inducer is higher in *tnrA* and *codY* mutants than in the wild-type strain, leading to a higher level of expression of the *bkd* operon in the mu-

TABLE 5. Effect of a *codY* mutation on the expression of the *bkd* operon

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein) in the following minimal medium:			
		MM + glucose	MM + glucose + isoleucine (20 mM)	MM + glucose + 0.2% Casamino Acids	MM + glucose + 0.2% Casamino Acids + isoleucine (20 mM)
QB7501	*ptb'-'lacZ*	20	1,975	45	575
QB7522	*ptb'-'lacZ codY*::*erm*	140	2,255	765	2,325

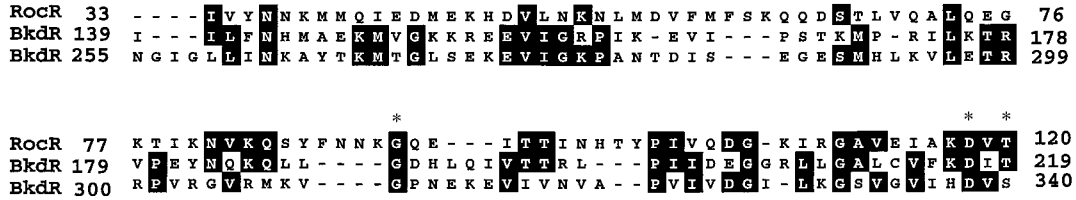

FIG. 6. Alignment of PAS-like domains of RocR and BkdR from *B. subtilis*. Conserved amino acid residues are boxed. Asterisks indicated the locations of constitutive missense mutations in RocR.

tants. Transcription of the operon at a high level requires the product of *bkdR*, a regulator with a central domain.

The different domains of members of this family of transcriptional activators have different functions and exhibit various degrees of evolutionary conservation (23). Typically, the amino-terminal domain is the signal reception domain of approximately 160 residues. There is only one known exception, the LevR protein of *B. subtilis*, in which the carboxy-terminal domain of the protein contains two signal reception domains (18). The amino-terminal part of BkdR is a large domain composed of 360 residues, a characteristic which is shared by two other regulators containing a central domain: AcoR from *Alcaligenes eutrophus* (9a) and AcoR from *B. subtilis* (10). This amino-terminal part contains two PAS-like domains (Fig. 6) (39). These domains, previously reported for proteins from mammals, insects, plants, fungi, and cyanobacteria, are involved in protein-protein interactions. They have been identified for a variety of sensor proteins that sense light, oxygen, and redox potential. In bacteria, the PAS domain is usually associated with the input domain of a histidine kinase or a sensor protein that regulates a histidine kinase. A PAS-like domain has also been found in the RocR regulator controlling arginine utilization in *B. subtilis* (39). Constitutive mutations located in the amino-terminal domain of RocR affect conserved residues of the PAS domain (3, 7), indicating that it is probably involved in the control of the activity of RocR. These PAS repeats may also be involved in the control of BkdR activity in response to the presence of the internal inducer. Site-directed mutagenesis of conserved residues in PAS domains of BkdR is under way as part of work to confirm their involvement in BkdR activity. Moreover, as nothing is known about the transcription of *bkdR*, further work is needed to define its regulation.

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