JOURNAL OF BACTERIOLOGY, Mar. 1993, p. 1392–1404
0021-9193/93/051392-13$02.00/0

Vol. 175, No. 5

Characterization of the Genes of the 2,3-Butanediol Operons from *Klebsiella terrigena* and *Enterobacter aerogenes*

KRISTINA BLOMQVIST,[1]† MATTI NIKKOLA,[1]‡ PÄIVI LEHTOVAARA,[1] MAIJA-LIISA SUIHKO,[1] ULLA AIRAKSINEN,[1] KERSTIN B. STRÅBY,[2] JONATHAN K. C. KNOWLES,[1]§ AND MERJA E. PENTTILÄ[1]*

Biotechnical Laboratory, Technical Research Center of Finland, P.O. Box 202, SF-02151 Espoo, Finland,[1] and Department of Microbiology, University of Umeå, S-90187 Umeå, Sweden[2]

Received 15 May 1992/Accepted 8 December 1992

The genes involved in the 2,3-butanediol pathway coding for α-acetolactate decarboxylase, α-acetolactate synthase (α-ALS), and acetoin (diacetyl) reductase were isolated from *Klebsiella terrigena* and shown to be located in one operon. This operon was also shown to exist in *Enterobacter aerogenes*. The *budA* gene, coding for α-acetolactate decarboxylase, gives in both organisms a protein of 259 amino acids. The amino acid similarity between these proteins is 87%. The *K. terrigena* genes *budB* and *budC*, coding for α-ALS and acetoin reductase, respectively, were sequenced. The 559-amino-acid-long α-ALS enzyme shows similarities to the large subunits of the *Escherichia coli* anabolic α-ALS enzymes encoded by the genes *ilvB*, *ilvG*, and *ilvI*. The *K. terrigena* α-ALS is also shown to complement an anabolic α-ALS-deficient *E. coli* strain for valine synthesis. The 243-amino-acid-long acetoin reductase has the consensus amino acid sequence for the insect-type alcohol dehydrogenase/ribitol dehydrogenase family and has extensive similarities with the N-terminal and internal regions of three known dehydrogenases and one oxidoreductase.

In some bacteria, pyruvate can be channeled via α-acetolactate into the neutral compound 2,3-butanediol, the production of which is enhanced when oxygen is limited and the pH is lowered (16, 22). It has been shown that when the pH is as low as 5.8, 55% of the pyruvate goes into 2,3-butanediol production and the enzymes of the pathway constitute 2.5% of total protein in *Aerobacter aerogenes* (16). The metabolic function of the 2,3-butanediol pathway is not yet known, although it might play a role in preventing intracellular acidification by changing the metabolism from acid production to the formation of neutral compounds (3). Consequently, acetic acid is able to induce the 2,3-butanediol pathway (4, 39). In addition, the 2,3-butanediol pathway might participate in regulation of the $NADH/NAD^+$ ratio in the bacteria (16, 22).

Three enzymes are involved in the 2,3-butanediol pathway (16, 17, 40): catabolic α-acetolactate synthase (α-ALS) (EC 4.1.3.18), α-acetolactate decarboxylase (α-ALDC) (EC 4.1.1.5), and acetoin (diacetyl) reductase (AR) (also called butanediol dehydrogenase) (Fig. 1). The catabolic α-ALS is involved in the formation of α-acetolactate from pyruvate. α-Acetolactate is decarboxylated by α-ALDC into acetoin, which in turn is reduced in a reversible reaction into 2,3-butanediol by AR. This enzyme is also involved in irreversible reduction of diacetyl to acetoin.

The term catabolic α-ALS is used to distinguish this enzyme from the anabolic α-ALS (also referred to as α-acetohydroxy acid synthase) involved in the valine-leucine and isoleucine pathways. The anabolic α-ALS isozymes have been widely studied and are encoded by the genetic loci *ilvBN*, *ilvGM*, and *ilvIH* in *Escherichia coli* and *Salmonella typhimurium* (7, 19, 33, 37, 44, 46). Two of these isozymes, coded by *ilvBN* and *ilvIH*, are feedback inhibited by the end product (19, 44). Regulation of the catabolic α-ALS enzyme has not been fully studied, and no comparison has been made between the genes encoding the anabolic and catabolic enzymes.

The use of bacterial species of the genera *Klebsiella*, *Enterobacter*, and *Bacillus* for production of 2,3-butanediol from waste from the pulp and cheese industries has received considerable attention. This compound could have a number of significant industrial applications, for instance, as an antifreeze agent or liquid fuel (reviewed in references 22 and 29). However, so far it has been difficult to develop an economically feasible process for bacterial 2,3-butanediol production.

In this article, we report the isolation and characterization of the butanediol operon coding for α-ALDC, α-ALS, and AR from *Klebsiella terrigena* and that the regulation of the operon occurs at the transcriptional level. In addition, we show that the gene encoding α-ALDC from *Enterobacter aerogenes* is also part of an operon.

MATERIALS AND METHODS

Strains and plasmids. The bacteria *K. terrigena* (*Aerobacter aerogenes*) VTT-E-74023 and *E. aerogenes* VTT-E-87292 were from the Technical Research Center of Finland (VTT) Collection of Industrial Microorganisms. *E. coli* HB101 (34) was used as a host for construction of the cosmid bank, and *E. coli* DH5α (Bethesda Research Laboratories) was used as a cloning host. *E. coli* MI262 (*leuB6 ilvI614 ilvH612* λ^- *relA1 spoT1 ilvB619 ilvG603 ilvG605*(Am) *thi-1*) (10), used in complementation studies, was provided by B. Bachmann (*E. coli* Genetic Stock Center, Yale University, New Haven, Conn.). Cosmid p3030 (described in reference 28) was used for gene bank construction. Bluescribe M13+ (Stratagene),

* Corresponding author.
† Present address: Department of Microbiology, University of Umeå, S-90187 Umeå, Sweden.
‡ Present address: Institute of Molecular Biology, S-75124 Uppsala, Sweden.
§ Present address: Glaxo Institute for Molecular Biology S.A., 1211 Geneva 24, Switzerland.

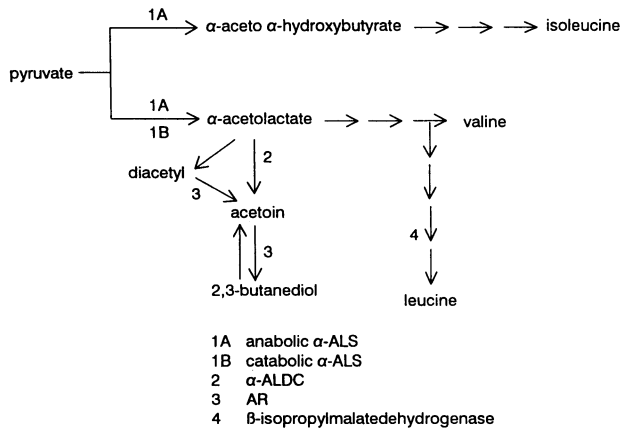


FIG. 1. Enzymes involved in the 2,3-butanediol pathway and in the isoleucine and valine-leucine pathways relevant to this study. In *E. coli*, the anabolic α-ALS isozymes are coded for by *ilvBN*, *ilvGM*, and *ilvIH* and the β-isopropyl malate dehydrogenase is coded for by the *leuB* gene.

pBluescript II SK M13+ (Stratagene), and pUC19 (Boehringer, Mannheim, Germany) were used in subcloning.

Media and growth conditions. Bacterial strains were cultivated in LB medium (24) at 37°C. Transformants were selected on LA plates (24) containing 100 μg of ampicillin per ml. The α-ALDC-active clones were selected on commercial Voges-Proskauer (VP) agar (2.5 g of bacteriological peptone, 1.0 g of yeast extract, 5 g of D-glucose, 1.0 g of sodium pyruvate [pH ~6.8], 25.0 g of agar A per liter) (Mast ID33), provided by Mast Laboratories Ltd. For α-ALDC activity measurements, the bacterial clones were cultivated in the same VP medium without agar but containing 100 μg of ampicillin per ml. For complementation tests, *E. coli* MI262 was cultivated on minimal medium (24) supplemented with 50 μg of isoleucine, 50 μg of leucine, and 100 μg of valine per ml.

Enzymes and chemicals. Restriction enzymes, T4 ligase, T4 polynucleotide kinase, calf intestinal phosphatase, deoxynucleotides, dideoxynucleotides, Klenow enzyme, and deoxy-7-deazaguanosine triphosphate were obtained from Boehringer and used as recommended by the manufacturer. Exonuclease III and S1 enzyme were provided by Boehringer and Bethesda Research Laboratories. Sequenase was obtained from United States Biochemical Corporation and used as described by the manufacturer. The diester α-acetolactic acid ethylester acetate used for α-ALDC enzyme activity measurements was provided by Oxford Organic Chemicals Ltd.

Preparation of cosmid gene bank. Chromosomal DNA from *K. terrigena* was isolated by the phenol extraction method described by Amundsen and Neville (1). The DNA was partially digested with *Sau*3A to obtain fragments of 30 to 40 kb, which were ligated to the *Bam*HI site of cosmid p3030. The resulting hybrid molecules were packaged in vitro into bacteriophage lambda particles by the method described by Hohn and Murray (14) and transduced into *E. coli* HB101.

Heterologous hybridization of *E. aerogenes* DNA. Chromosomal DNA was isolated by the method of Amundsen and Neville (1) and digested with different restriction enzymes, and 400 ng of the digests was blotted onto a GeneScreen filter (NEN Research Products) for Southern hybridization. Hybridization was carried out at 35°C in 40% formamide–6× SSC (0.9 M NaCl, 90 mM sodium citrate [pH 7.0])–10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin)–0.5% sodium dodecyl sulfate–100 μg of herring sperm DNA per ml–10 μg of poly(A) per ml overnight with a probe labeled to a specific activity of $>10^8$ cpm/μg with [α-^{32}P]dCTP. After hybridization, the filters were washed at 42°C, three times with 6× SSC and three times with 2× SSC.

Northern (RNA blot) hybridization. RNA was isolated (43) from *K. terrigena* and *E. aerogenes* grown in VP broth to an exponential growth phase. Ten micrograms of RNA was glyoxylated (23), run in an agarose gel, and blotted onto a Hybond-N filter (Amersham). Hybridization was carried out as described by the manufacturer with a probe labeled with [α-^{32}P]dCTP.

Sequencing. Plasmid pB5, which contains the *budA*, *budB*, and most of the *budC* genes on a 3.2-kb partial *Sau*3A fragment in Bluescribe M13+, was cut in the polylinker sequences with either *Asp* 718 plus *Sac*I or *Bam*HI plus *Sph*I and treated with exonuclease III and S1 enzyme by the method of Henikoff (13). Sequences were determined from denatured double-stranded plasmids (47) by the modified dideoxy method (32). Deoxy-7-deazaguanosine triphosphate was used instead of dGTP to avoid formation of compressed areas (25). Both commercially available vector-specific primers (Boehringer) and gene-specific primers (synthesized by M. Leppäkoski, University of Helsinki, Finland) were used.

VP test. *E. coli* transformants were replicated from LA plates to VP agar plates. α-ALDC-active clones were selected by pouring onto the incubated plates a mixture containing 2.5% agarose (Indubiose A37) and 0.8% α-naphthol (5% stock solution in 2.5 N NaOH). The development of a red color (6, 45) was monitored for 1 h at room temperature.

The VP test of liquid bacterial cultures grown in VP medium containing 100 μg of ampicillin per ml was carried out by adding 300 μl of freshly prepared 5% α-naphthol in absolute ethanol and 100 μl of 40% KOH to 1 ml of overnight culture. The mixture was stirred vigorously, and the formation of red color was monitored for 30 min at room temperature. The VP test for α-ALDC enzyme activity was carried out as follows: 500 μl of 0.3% creatin, 600 μl of freshly prepared 5% α-naphthol in absolute ethanol, and 300 μl of 40% KOH were added to 1.2 ml of bacterial sample mixture (see α-ALDC activity measurements below). Formation of red color was monitored for 30 min, and the A_{540} was measured.

α-ALDC activity measurements. Bacteria were grown in 10 ml of VP medium overnight at 37°C without shaking. Cells were washed twice with 0.1 M phosphate buffer (pH 7.0) and resuspended in 5 ml of the same buffer. Lysozyme (5 mg for *E. coli* and 10 mg for *K. terrigena* and *E. aerogenes*) was added, and the suspension was incubated at 37°C for 30 min. The suspension was examined under a microscope, and if the cells were not completely broken, they were sonicated 10 times for 15 s each. Cell debris was pelleted by centrifugation, and 80 μl of freshly hydrolyzed (27a) α-acetolactate substrate was added to 1 ml of the supernatant (or dilutions thereof). Incubation was carried out at 30°C for 60 min. The reaction was stopped by adding 200 μl of 1 N NaOH, and the mixture was transferred to ice. The amount of acetoin formed was determined by the VP test (see above) or gas chromatography (41).

Primer extension. Total RNA was isolated from *K. terrigena* and *E. coli* containing pPL2 (Fig. 2) as described above

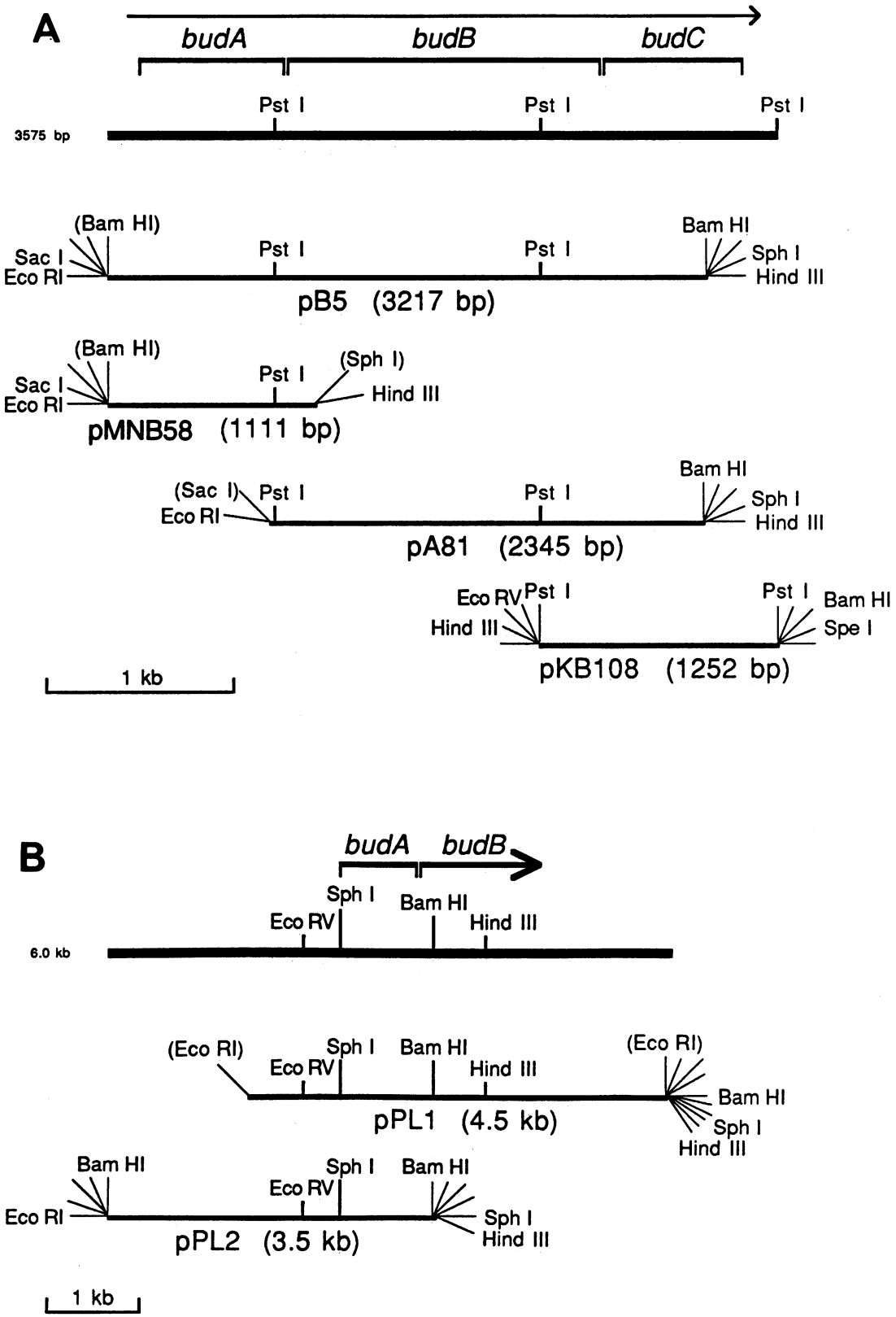


FIG. 2. *K. terrigena* (A) and *E. aerogenes* (B) inserts carried by the plasmids indicated. The *K. terrigena* genes were cloned into the multiple cloning site of Bluescribe M13+ (pB5, pMNB58, and pA81) and of pBluescript II SK M13+ (pKB108). The *E. aerogenes* genes were cloned into the multiple cloning site of pUC19. Plasmids pMNB58 and pA81 result from deletions of the *K. terrigena* insert in pB5 from each end. The exact (A) or approximate (B) size of the insert is shown in parentheses after the plasmid name.

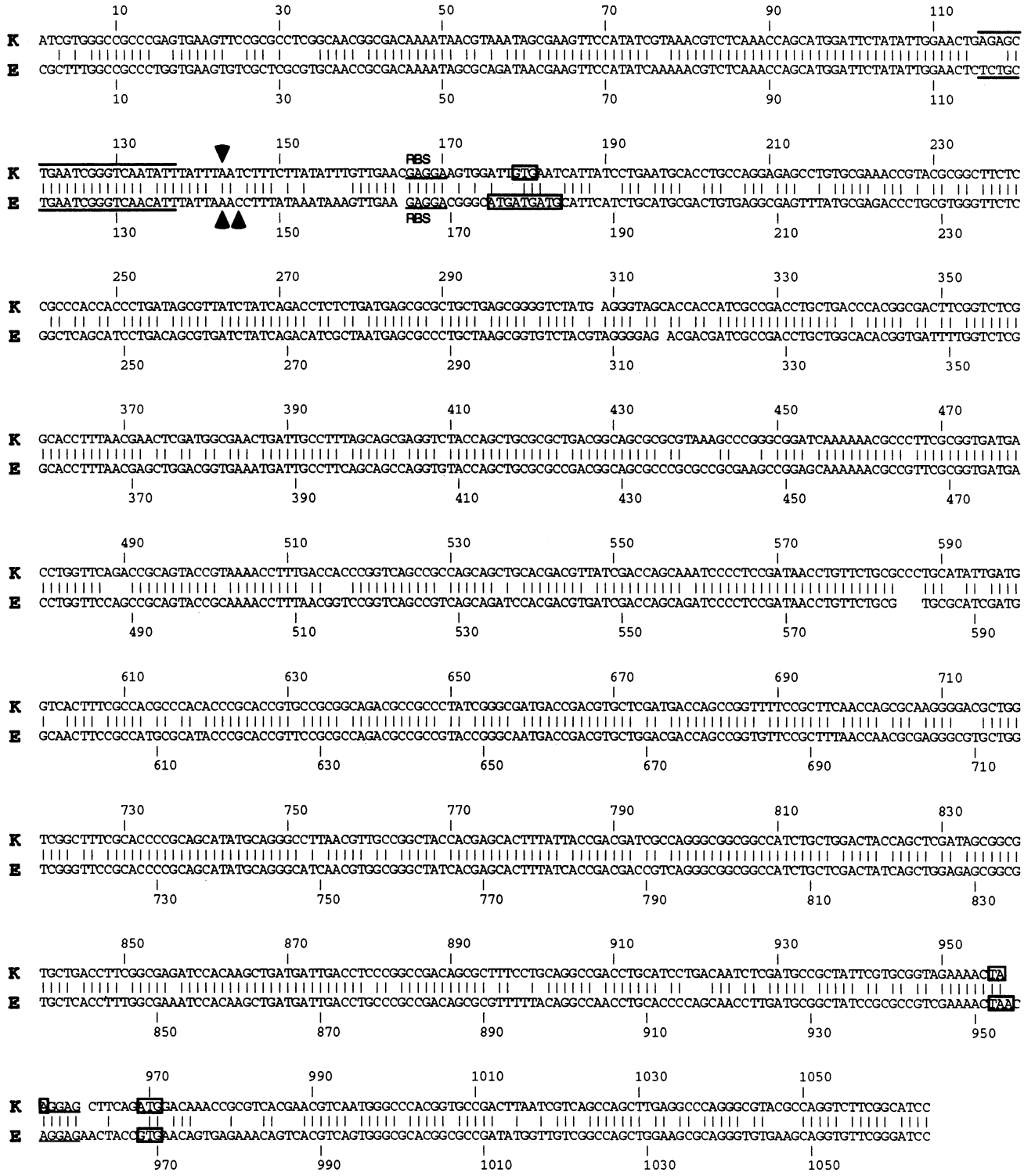


FIG. 3. Comparison of the nucleotide sequences of the *K. terrigena* (K) and *E. aerogenes* (E) *budA* genes and flanking areas. The transcriptional start sites are marked with arrowheads. A sequence with homologies to an FNR-binding site (36) is indicated upstream of the transcriptional start site. Relevant start and stop codons are boxed, and putative ribosome-binding sites (RBS) are underlined. The start codons for the coding regions of the *budB* genes are also boxed.

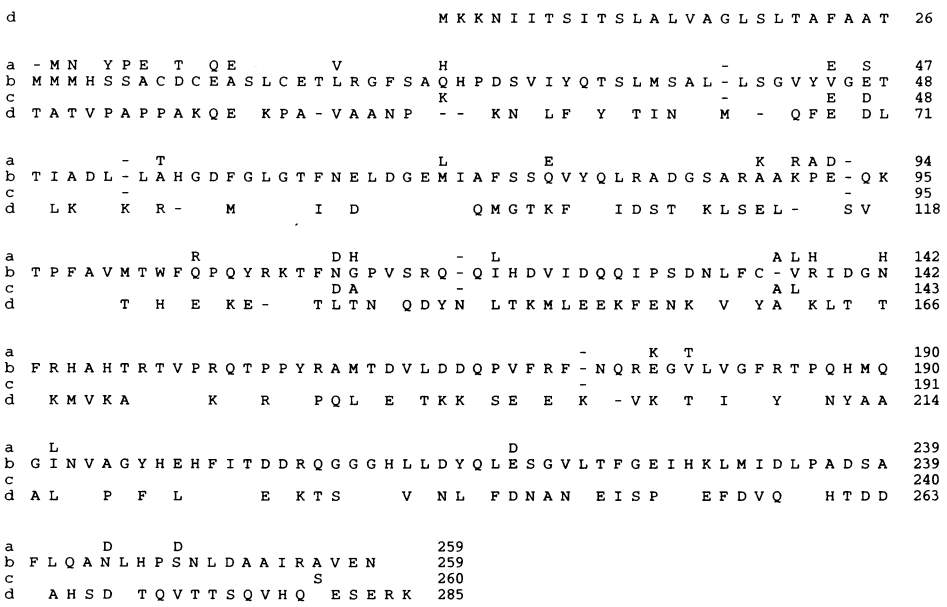


FIG. 4. Alignment of the amino acid sequences of the *K. terrigena* (a) and *E. aerogenes* (b) α-ALDC enzymes determined in this study and the sequence of the enzyme from *E. aerogenes* IFO 13534 (c) (35) and *B. brevis* (d) (5). Only amino acids that differ from those in the *E. aerogenes* sequence obtained in this study (b) are shown for the three other sequences. Gaps (—) were inserted in the sequences to obtain maximal alignment.

under Northern (RNA blot) hybridization. The transcription start site was identified by using the Primer Extension System from Promega, and adding 10 μg of RNA and 100 ng of sequence-specific primer to the reaction mix.

AR activity. *E. coli* DH5α containing plasmid pKB108 was grown in 5 ml of LB medium overnight at 37°C. The cells were harvested at 4,000 rpm (10 min), washed once in 50 mM potassium phosphate (pH 7.0), recentrifuged and dissolved in 5 ml of 50 mM potassium phosphate (pH 7.0) containing 50 mg of lysozyme, and incubated at 37°C for 30 min. Cell debris was separated from the cell extract by centrifugation at 4,000 rpm for 10 min; then 0.1 ml of 500 mM potassium phosphate (pH 7.0), 0.1 ml of 1 mM NADH, and 0.1 ml of 50 mM acetoin were added to 0.7 ml of cell extract. Oxidation of NADH was measured at 340 nm after 60 min of incubation at room temperature. Cell extract, prepared as described above, from *K. terrigena* grown in 5 ml of VP medium under semianaerobic conditions was used as a positive control.

Slot-blot hybridization. Total RNA was prepared from cells in logarithmic growth phase as described above under Northern (RNA blot) hybridization. *K. terrigena* was grown with shaking in 5 ml of LB or with or without (semianaerobic) shaking in 5 ml of VP medium. Total RNA prepared from *E. coli* DH5α grown with shaking in LB was used as a negative control. From 2.5 to 20 μg of RNA was added to a Hybond-N filter (Amersham) with a slot-blot apparatus. Hybridization was carried out as described previously.

Nucleotide sequence accession numbers. The DNA sequences from *E. aerogenes* and *K. terrigena* were deposited

TABLE 1. Activity of different *K. terrigena* and *E. aerogenes* clones

Strain	Plasmid	Origin of plasmid-borne gene(s)	Gene present		VP test reaction	α-ALDC activity	Complementation of defect in *E. coli* MI262[a] in medium containing:			
			budA	*budB*			Leucine	Valine + leucine	Isoleucine + leucine	Isoleucine + valine + leucine
E. coli DH5α	pB5	*K. terrigena*	+	+	+	+	−	−	(+)	++
	pMNB58	*K. terrigena*	+	−	−	+	−	−	−	++
	pA81	*K. terrigena*	−	+	+	−	−	−	+	++
	pPL1	*E. aerogenes*	+	+	+	+	−	−	−	++
	pPL2	*E. aerogenes*	+	−	−	+	−	−	−	++
Control strains										
E. coli DH5α			−	+[b]	−	−	+	(+)	+	++
K. terrigena			+	+	+	+				
E. aerogenes			+	+	+	+				
E. coli MI262			−	−	ND[c]	ND	−	−	−	++

[a] Complementation of the valine deficiency of mutant *E. coli* MI262. Strains were grown on minimal medium supplemented with the amino acids indicated. Leucine was added because strain MI262 also carries a *leuB* mutation. Growth was scored as good, very good, poor, or none [+, ++, (+), and −, respectively].

[b] Genes coding for anabolic α-ALS.

[c] ND, not determined.

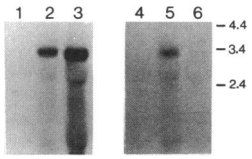


FIG. 5. Northern blot analysis of total RNA isolated from *E. coli* DH5α (lanes 1 and 4), *K. terrigena* (lanes 2 and 5), and *E. aerogenes* (lanes 3 and 6). Lanes 1 to 3 were hybridized with the *budA* gene-containing fragment of *K. terrigena* (*Eco*RI-*Hin*dIII fragment of pMNB58, Fig. 2A) and *E. aerogenes* (*Eco*RV-*Hin*dIII fragment of pPL2, Fig. 2B). Lanes 4 to 6 were hybridized with the *budB* gene-containing fragment of *K. terrigena* (*Eco*RI-*Hin*dIII fragment of pA81, Fig. 2A). Sizes are shown in kilobases.

in GenBank and given accession numbers L04506 and L04507, respectively.

RESULTS

Cloning of the *budA* gene encoding α-ALDC from *K. terrigena* and *E. aerogenes*. As *E. coli* does not possess an α-ALDC enzyme, the *K. terrigena* gene bank was established in *E. coli*. The bank was screened for expression of the *K. terrigena* gene by searching for clones giving a positive VP reaction in a plate assay. In this method, the acetoin formed by the α-ALDC enzyme is oxidized in very alkaline conditions to diacetyl, which in turn reacts with the guanidine group of creatine and gives a strong red color in the presence of α-naphthol (6).

Clones that were positive in the VP test were tested further for α-ALDC activity with α-acetolactate as the substrate. Gas chromatographic analysis showed that in cell extracts of active *E. coli* clones, the added α-acetolactate was converted directly to acetoin (data not shown). This indicated that these cosmid clones carried and expressed the *budA* gene of *K. terrigena*.

The gene was subcloned, and the size of the insert in one of the VP-positive clones (pB5, Fig. 2A) was further decreased by deletions from both ends. The α-ALDC activity of these deletion clones was tested, and the region giving rise to activity was sequenced on both strands from overlapping deletion clones. The longest open reading frame found coded for a protein of 259 amino acids, with GTG as the start codon. The deletion clone pMNB58 (Fig. 2A) carried this entire open reading frame.

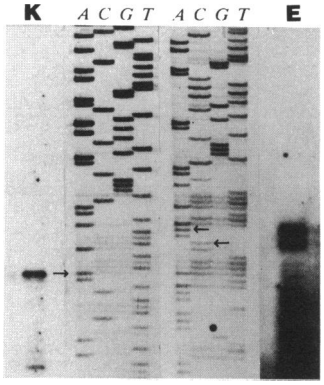


FIG. 6. Primer extension to locate the transcriptional start of the *budABC* operon of *K. terrigena* (K, left side) and *E. aerogenes* (E, right side). Transcription start sites are indicated by arrows in the DNA sequence obtained with the same primer as used in the primer extension procedure.

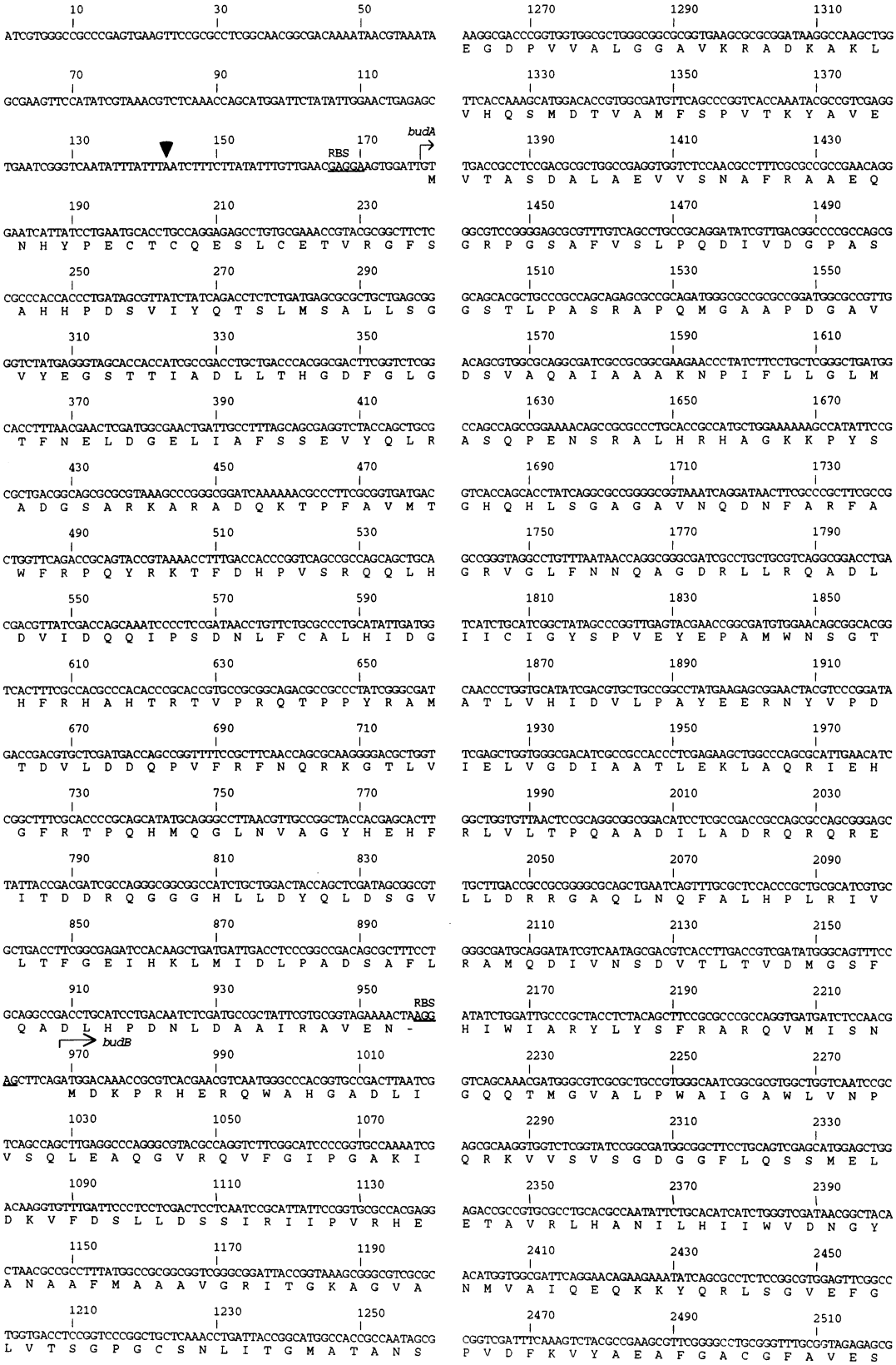
The gene coding for α-ALDC was also cloned from an *E. aerogenes* strain. The previously isolated *K. terrigena budA* gene was shown to hybridize to total *E. aerogenes* DNA in optimized hybridization conditions. DNA fragments giving single hybridization bands in Southern analysis were cloned into the vector pUC19, giving plasmids pPL1 and pPL2 (Fig. 2B). Sequencing of pPL2 gave an open reading frame with strong homology to the *budA* gene of *K. terrigena*, which was thus tentatively identified as the *budA* gene of *E. aerogenes*.

Structure of *budA* genes and homology of the proteins. The amino acid sequences of α-ALDC deduced from the nucleotide sequences of the *K. terrigena* and *E. aerogenes* clones (Fig. 3 and 4) show 87% similarity throughout the putative protein-coding region. This strongly suggests that in both organisms, α-ALDC is a 259-amino-acid-long protein consisting of the amino acids shown in Fig. 4. Furthermore, the previously published sequence of the gene coding for α-ALDC from another strain of *E. aerogenes* (35) codes for a protein which is 88 and 97% similar to the *K. terrigena* and *E. aerogenes* sequences, respectively, obtained in this study. The nucleotide sequence of a gene from *Bacillus brevis* encoding a secreted α-ALDC has also been published (5). This enzyme is 43 and 34% similar in amino acid sequence to the *K. terrigena* and *E. aerogenes* enzymes, respectively, studied here. The four α-ALDC protein sequences are compared in Fig. 4.

Localization and characterization of the genes coding for α-ALS. One of the clones, pMNB58, now known to carry the *budA* gene of *K. terrigena* (Fig. 2A), was negative in the VP test. However, it showed activity in the enzyme assay when the substrate α-acetolactate was added to the cell extract. This suggests that the amount of the substrate, α-acetolactate, is naturally too low in *E. coli* cells to result in sufficient acetoin formation by α-ALDC to allow detection.

Interestingly, the original cosmid clone as well as subclone pB5 (Fig. 2A) were positive in the VP test without the addition of α-acetolactate. These clones carry additional *K. terrigena* DNA besides the *budA* gene. The deletion clone pA81 (Fig. 2A), derived from pB5 and lacking the *budA* sequence, gave an immediate strongly positive reaction in the VP test (Table 1). Similar results were also obtained with *E. aerogenes* clones. Cells harboring plasmid pPL1 (Fig. 2B) were positive in the VP test, whereas cells carrying plasmid pPL2 (Fig. 2B) showed activity only if α-acetolactate was added to the enzyme activity assay mixture (Table 1). As α-acetolactate is spontaneously decarboxylated into diacetyl, a positive VP reaction is expected if excess α-acetolactate is produced in the cells. Thus, our results indicated that we had also cloned the gene coding for an α-ALS.

In *K. terrigena* and *E. aerogenes*, the catabolic α-ALS enzyme converts pyruvate to α-acetolactate, which is an intermediate in the valine and leucine pathway, but unlike the anabolic α-ALS enzyme, it is not involved in the formation of isoleucine (Fig. 1). To determine whether the *K. terrigena* gene present in our clones was coding for catabolic or anabolic α-ALS, mutant *E. coli* MI262, which is deficient in both the leucine-valine and isoleucine pathways because it lacks the anabolic α-ALS isozymes, was transformed with the *K. terrigena* plasmids described above and tested for complementation. Plasmids carrying only the *budA* gene failed to complement the mutation, whereas the *K. terrigena* clone containing the putative gene for α-ALS gave successful complementation when isoleucine and


budA
RBS
budB

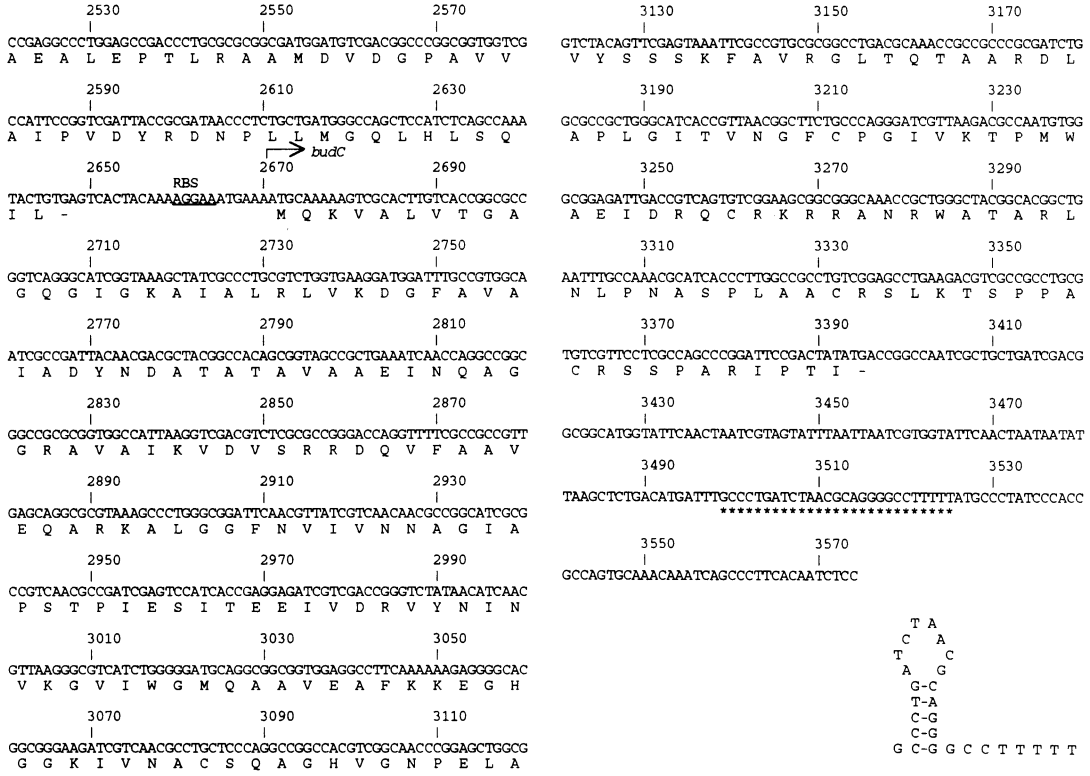


FIG. 7. Complete nucleotide sequence of the *budABC* operon of *K. terrigena*. The transcription start site is marked with an arrowhead. The amino acids encoded by the three open reading frames are shown below the nucleotide sequence. Putative ribosome-binding sites (RBS) are underlined. A potential transcription stop signal is underlined with asterisks and shown as a loop structure at the end.

leucine were added to the medium (Table 1). On the other hand, complementation was not obtained when valine was added instead of isoleucine. Thus, it seemed that the gene located 3′ to the *budA* gene in *K. terrigena* codes for the catabolic α-ALS. This gene was called *budB*. Clones carrying both the *budA* and *budB* genes did not complement the mutation or complemented it only poorly. This retention of the auxotrophy could be due to α-ALDC activity, which turns the α-acetolactate formed by α-ALS into acetoin and consequently removes it from the leucine-valine pathway.

To determine whether the *budA* and *budB* genes were located in the same operon, Northern analysis of RNA isolated from *K. terrigena* and *E. aerogenes* was carried out. In *K. terrigena*, probes containing the *budA* or *budB* gene hybridized to the same band of about 3.4 kb (Fig. 5), showing that *budA* and *budB* are transcribed into a common mRNA. This is also the case in *E. aerogenes*, since the probe carrying *budA* sequences hybridized to a band of approximately the same size (about 3.4 kb). Primer extension data revealed a transcription start site at an A at position −36 with respect to the translational start site of *budA* in the operon of *K. terrigena*. In the 5′ region of the *budA* gene from *E. aerogenes*, two transcription start sites were found, one at an A at position −33 and the other at a C at position −31 with respect to the translational start site (Fig. 3 and 6).

Sequencing of *budB* from *K. terrigena* revealed an open reading frame of 1,677 nucleotides, encoding a protein of 60 kDa (Fig. 7). The amino acid sequence showed similarities of 26, 26, and 24% to the large subunits of the anabolic α-ALS isozymes of *E. coli* referred to as AHAS I, II, and III, respectively (7, 19, 37, 46), encoded respectively by *ilvB*, *ilvG*, and *ilvI* (Fig. 8). In the *E. aerogenes* strain studied here, the *budA* gene is also followed by a putative ribosome-binding site and an open reading frame, *budB* (Fig. 3), whose putative product showed amino acid similarity to the three anabolic α-ALS isozymes of *E. coli* (Fig. 8). Sequence comparisons suggest that the gene encoding α-ALDC in the *E. aerogenes* strain studied by Sone et al. (35) is also followed by a gene encoding α-ALS.

Gene coding for AR. The third enzyme in the butanediol pathway, AR, purified from *E. aerogenes* is composed of four equal-size subunits of 25 kDa (40). The 3.4-kb-long *K. terrigena* mRNA, in which the *budAB* transcript covers 2.5 kb, would still have space within the mRNA for a protein of this size.

Nucleotide sequencing revealed an additional open reading frame 3′ of the *K. terrigena budB* gene which seemed to extend beyond the *K. terrigena* sequence present in plasmid pB5. For this reason, missing sequences were cloned from total *K. terrigena* DNA digested with *Pst*I. A 1.2-kb fragment which hybridized with the probe specific for the 3′-most *K. terrigena* sequence in pB5 was cloned, giving plasmid pKB108 (Fig. 2A), and the insert was sequenced.

The open reading frame of 729 bp present in plasmid pKB108 corresponds to a protein of 25.6 kDa (Fig. 7). In this gene, named *budC*, there are two potential start codons in frame. The second ATG is better positioned with respect to a putative ribosome-binding site (Fig. 7). The deduced amino acid sequence showed similarities, particularly in the N-terminal region but also in some internal areas, to three known dehydrogenases, 15-hydroxyprostaglandin dehydrogenase

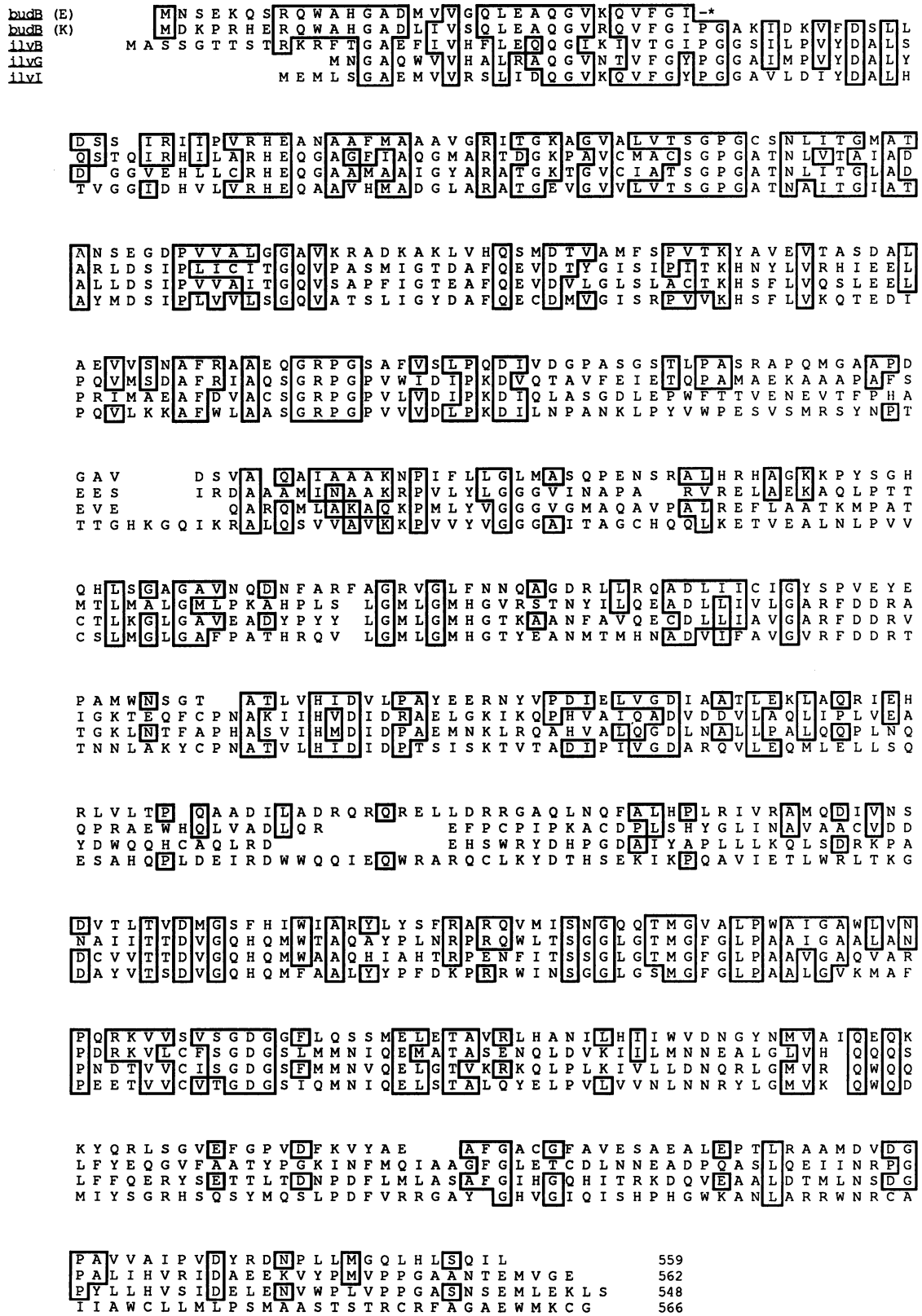


FIG. 8. Alignment of the amino acid sequence deduced from the *budB* gene of *K. terrigena* (K) and the amino acid sequences of the large subunits of the anabolic α-ALS isozymes coded by the *ilvB*, *ilvG*, and *ilvI* genes of *E. coli*. The first N-terminal amino acids deduced from the putative *budB* gene (see Fig. 3) of *E. aerogenes* (E) are also shown at the top. Amino acids identical to those in the *K. terrigena* *budB*-encoded protein are boxed. Gaps were introduced to obtain maximal alignment. The total number of amino acids in the protein is indicated at the end of the sequence. *, Only the N-terminal sequence is available.

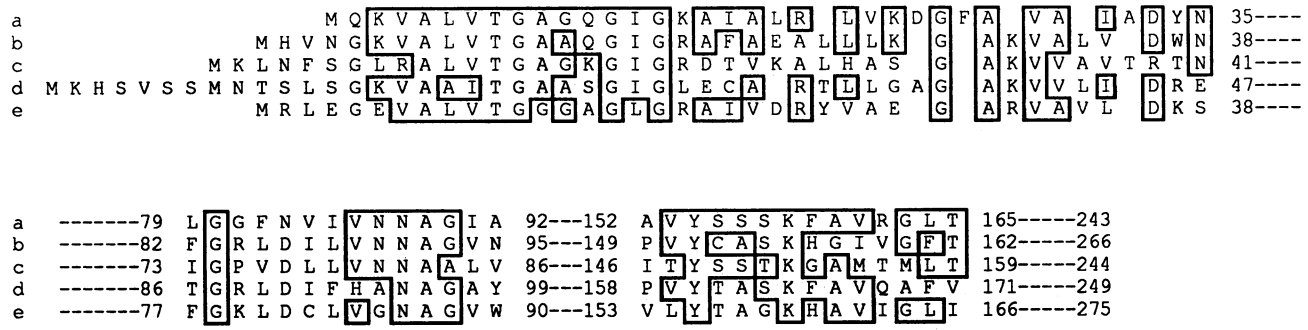


FIG. 9. Alignment of the amino acid sequences in the N-terminal and two internal regions of the protein coded by the *budC* gene from *K. terrigena* (a) and of 15-hydroxy prostaglandin dehydrogenase from *Homo sapiens* (b), adipocyte P27 protein from *Mus musculus* (c), ribitol dehydrogenase from *Klebsiella aerogenes* (d), and *cis*-benzene glycol dehydrogenase from *Pseudomonas putida* (e). Amino acids identical to those in the *K. terrigena* sequence are boxed. Gaps were introduced to obtain maximal alignment. The numbers nearest to an amino acid refer to its position in the protein, and the total number of amino acids in the protein is shown at the end. Dashes (—) indicate a region of protein that is not shown. The consensus pattern Y-(S, T, A, G, or C)-(S, T, A, G, C, or V)-(S, T, A, G, or C)-K-X-(A or G)-(L, I, V, M, A, or G)-X-X-(L, I, V, M, or F) for the insect-type alcohol dehydrogenase/ribitol dehydrogenase family is situated at amino acids 154 to 164 in the *K. terrigena* sequence.

(18), ribitol dehydrogenase (21), and *cis*-benzene glycol dehydrogenase (15), and also to the oxidoreductase adipocyte P27 (26) (Fig. 9).

A cell extract of *E. coli* DH5α containing plasmid pKB108 was tested for AR activity by addition of acetoin and the cofactor NADH. Comparisons were made with cell extracts of *E. coli* harboring the vector without an insert and of *K. terrigena* (Fig. 10). After 60 min of incubation, no AR activity, measured as a decrease in A_{340}, was detected in *E. coli* cells carrying the vector. However, the cell extract of *E. coli* carrying pKB108 had AR activity, although the level was low compared with that in the cell extract from *K. terrigena* (Fig. 10). The rather low levels of AR found in cells carrying pKB108 are most likely the result of there being no known promoter sequences upstream of the *budC* gene in pKB108 (Fig. 2A).

***budABC* operon regulated at the transcriptional level.** A slot-blot analysis of total RNA showed that the amount of *budABC* mRNA was high when *K. terrigena* was grown in VP medium, a medium especially designed for 2,3-butanediol production (Fig. 11), and very low when cells were grown in LB medium. The highest amount of transcript was seen when *K. terrigena* was grown under semianaerobic conditions in VP medium (Fig. 11). This demonstrates that the operon is regulated at the transcriptional level.

DISCUSSION

We show that the three enzymes involved in the bacterial synthesis of 2,3-butanediol from pyruvate are encoded by genes located in one operon in *K. terrigena* and *E. aerogenes* and are transcribed in the order *budA*, *budB*, and *budC*, encoding α-ALDC, α-ALS, and AR, respectively. No further reading frame was found, and a potential transcription terminator is located 3′ of the *budC* gene (Fig. 7). All three genes have a potential ribosome-binding site 6 to 7 nucleotides in front of the translational start site. The size of the operon is 3.4 kb, based on the nucleotide sequence, which agrees well with the size of the mRNA obtained in Northern analysis.

Optimal production of 2,3-butanediol is obtained under slightly acidic (pH 6), oxygen-limited conditions and in the presence of acetate (4, 16, 22). Thus, pyruvate is channeled into 2,3-butanediol production when the acetate in the medium reaches a critical concentration, and consequently 2,3-butanediol production prevents acetate overproduction and subsequent intracellular acidification. Since the first enzyme synthesized from the operon is α-ALDC and not α-ALS, a rapid change in the metabolism of the cell towards 2,3-butanediol production would occur at the expense of valine-leucine synthesis. In addition, reduction of acetoin to 2,3-butanediol serves to regenerate NAD^+ from NADH, and because this reaction is reversible, AR also permits regeneration of NADH. Thus, it seems that the 2,3-butanediol pathway serves to maintain the intracellular NAD^+-NADH balance in changing culture conditions (16, 22).

The *budA* genes of *K. terrigena* and *E. aerogenes* both code for a protein of 29.2 kDa, which corresponds well to the previously reported size of the purified α-ALDC protein of *Brevibacterium acetylicum* (27) and *Lactobacillus casei* (31). The α-ALDC protein has also been purified from *A. aerogenes* (20). The two α-ALDC enzymes studied here are very similar to each other at both the nucleotide level (82.4%) and the amino acid level (87%) and also to the previously published sequence of α-ALDC from another *E. aerogenes* strain (35). Interestingly, the *Bacillus brevis* α-ALDC (5) is only about 40% similar to the other α-ALDC enzymes characterized, and furthermore, this protein carries a typical N-terminal signal sequence and seems to be secreted from *B. brevis*. The *Streptococcus lactis* (8) and *Acetobacter pasteurianus* (38) α-ALDC genes have also been cloned, but no nucleotide or amino acid data are available yet.

The two α-acetolactate-forming enzymes, the anabolic one (also called the pH 8 enzyme) and the catabolic one (pH 6 enzyme), are synthesized under different culture conditions (9, 11). The anabolic α-ALS is involved in valine-leucine and isoleucine synthesis; it catalyzes the formation of both α-acetolactate and α-aceto-α-hydroxybutyrate from pyruvate. Genes for the three isozymes of the anabolic α-ALS, encoded by *ilvBM*, *ilvGN*, and *ilvIH*, from *E. coli* have been isolated and sequenced. The *ilvGMEDA* operon of *Klebsiella aerogenes* has also been cloned, but only the sequence of the regulatory regions is presently available (12). The anabolic α-ALS enzymes of *E. coli* consist of large subunits of 59, 60, and 62 kDa (encoded by *ilvG*, *ilvB*, and *ilvI*, respectively) and small subunits of 11, 10, and 18 kDa (encoded by *ilvM*, *ilvN*, and *ilvH*, respectively). This report

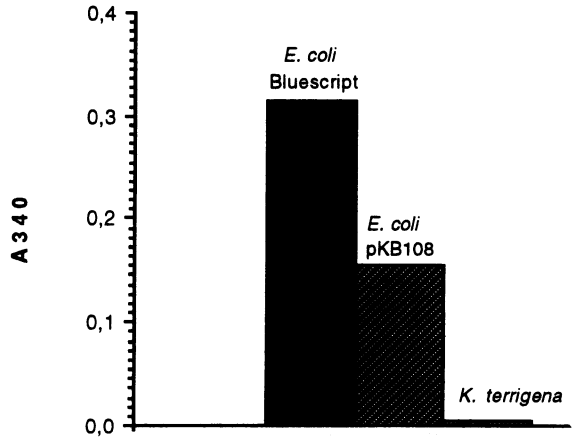


FIG. 10. AR activity was measured as oxidation of NADH to NAD^+, shown as reduction in A_{340}, after 60 min of incubation. The enzyme extracts were prepared from the strains indicated above the bars.

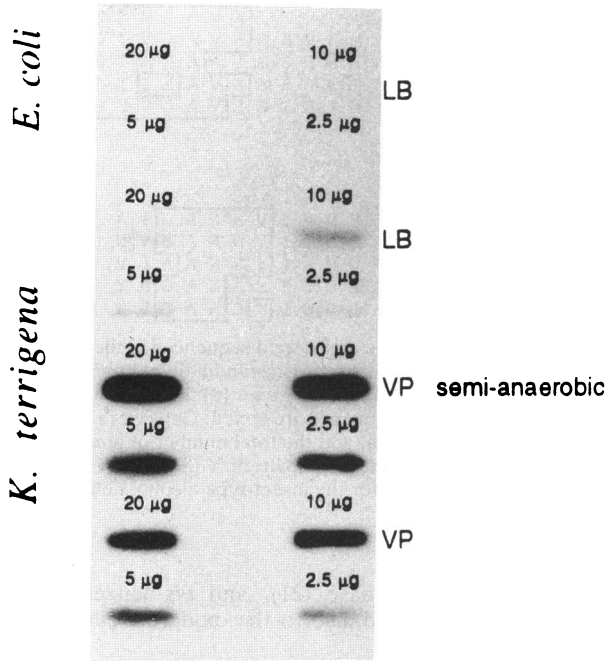


FIG. 11. Amounts of *budABC* transcript, analyzed by slot-blot hybridization, from *E. coli* grown in LB (top two slots in each lane) and from *K. terrigena* (bottom six slots in each lane) grown under different cultivation conditions as indicated on the right (four RNA concentrations per condition). The amount of RNA is indicated above the slots.

is the first one describing the cloning of a gene encoding the catabolic (pH 6) α-acetolactate-forming enzyme. The size of the *K. terrigena* α-ALS enzyme is 60 kDa, similar to that reported for the subunits of the purified dimeric catabolic α-ALS of *A. aerogenes* (40). Unlike the anabolic α-ALS enzymes of *E. coli*, the dimeric catabolic α-ALS of *A. aerogenes*, and most likely also that of *K. terrigena*, consists of only one type of subunit. Interestingly, the *K. terrigena* α-ALS studied here shows homology to the large subunits of the three anabolic α-ALS isozymes of *E. coli*, especially in the N-terminal regions (Fig. 8). We show here that the catabolic α-ALS enzyme of *K. terrigena* is able to convert pyruvate to α-acetolactate when expressed in *E. coli*, but it does not seem to be involved in the formation of α-aceto-α-hydroxybutyrate. It is tempting to speculate that the small subunits of the anabolic α-ALS isozymes have evolved especially for the formation of α-aceto-α-hydroxybutyrate.

The third enzyme in the 2,3-butanediol pathway, AR, purified from *A. aerogenes* consists of four equal-size subunits of 25 kDa (40). The last open reading frame (gene *budC*) in the *K. terrigena* operon codes for a protein of 25.6 kDa. The amino acid sequence shows similarity to that of three dehydrogenases (15, 18, 21), all of which contain a consensus pattern [Tyr-(Ser, Thr, Ala, Gly, or Cys)-(Ser, Thr, Ala, Gly, Cys, or Val)-(Ser, Thr, Ala, Gly, or Cys)-Lys-X-(Ala or Gly)-(Leu, Ile, Val, Met, Ala, or Gly)-X-X-(Leu, Ile, Val, Met, or Phe)] found in the insect-type alcohol dehydrogenase/ribitol dehydrogenase family. Such a consensus sequence was found in the *K. terrigena* AR at amino acids 154 to 164 (Tyr-Ser-Ser-Ser-Lys-Phe-Ala-Val-Arg-Gly-Leu) (Fig. 9). AR catalyzes the reversible formation of 2,3-butanediol from acetoin and thus works as both a reductase and a dehydrogenase. These sequence data, together with the demonstration of enzyme activity in *budC*-containing *E. coli* strains, cause us to conclude that *budC* encodes AR.

From our data, we conclude that the operon is regulated at the transcriptional level and that induction is improved by limited-oxygen conditions. The protein FNR is known to be a transcriptional activator of genes involved in anaerobic respiratory processes (36). In the 5′ region of the *budABC* operon in both *K. terrigena* and *E. aerogenes*, a sequence was found with similarities to an FNR-binding site (36). Interestingly, the putative FNR site found in the *budABC* operon is located at position −6, while most other operons activated by FNR have an FNR-binding site immediately 5′ of the −35 region. Furthermore, the *budABC* operon has no obvious consensus −35 region 5′ of the transcriptional start site, a feature characteristic of positively regulated operons (30). Thus, one way of regulating the operon might rest on the polymerase interacting with an FNR-like protein bound to DNA rather than with classical promoter regions only.

The cloning of the genes involved in the bacterial butanediol pathway now opens the possibility of elucidating the regulation of 2,3-butanediol production and its connection to the regulation of amino acid biosynthesis, pH, and anaerobiosis. It also makes possible the use of genetic engineering to improve 2,3-butanediol production as well as other biotechnical processes of interest. For instance, the *budA* gene (also called *α-ald* in references 2, 41, and 42) encoding α-ALDC has been transferred to brewer's yeast (2, 35, 41, 42). By using such recombinant strains in beer production, the conventional brewing process can be shortened remarkably, since the formation of diacetyl in fermenting wort is significantly reduced.

ACKNOWLEDGMENTS

We are grateful to T.-M. Enari for many useful discussions. The excellent technical assistance of Pirkko Veijola-Bailey, Kariitta Berg, Helena Hakuli, Riitta Nurmi, and Hanna Lehtonen is gratefully acknowledged.

This study was supported by Oy Panimolaboratorio-Bryggerilaboratorium AB, Technology Development Center of Finland (TEKES), Technical Research Center of Finland (VTT), Stiftelsen Svensk Etanolutveckling, and Swedish Natural Science Research Council (grant 2708).

REFERENCES

1. **Amundsen, S. K., and M. E. Neville.** 1979. Comparison of three procedures for isolating DNA from bacteria. Microbios **24**:29–39.
2. **Blomqvist, K., M.-L. Suihko, J. K. C. Knowles, and M. E. Penttilä.** 1991. Chromosomal integration and expression of two bacterial α-acetolactate decarboxylase genes in brewer's yeast. Appl. Environ. Microbiol. **57**:2796–2803.
3. **Booth, I. R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**:356–378.
4. **Brown, T. D. K., C. R. S. Periera, and F. C. Størmer.** 1972. Studies of the acetate kinase-phosphotransacetylase and the butanediol-forming systems in *Aerobacter aerogenes*. J. Bacteriol. **112**:1106–1111.
5. **Diderichsen, B., U. Wedsted, L. Hedegaard, B. R. Jensen, and C. Sjöholm.** 1990. Cloning of *aldB*, which encodes α-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J. Bacteriol. **172**:4315–4321.
6. **Eddy, B. P.** 1961. The Voges-Proskauer reaction and its significance: a review. J. Appl. Bacteriol. **24**:27–41.
7. **Friden, P., J. Donegan, J. Mullen, P. Tsui, M. Freundlich, L. Eoyang, R. Weber, and P. M. Silverman.** 1985. The *ilvB* locus of *Escherichia coli* K-12 is an operon encoding both subunits of acetohydroxy acid synthase I. Nucleic Acids Res. **13**:3979–3993.
8. **Goelling, D., and U. Stahl.** 1988. Cloning and expression of an α-acetolactate decarboxylase gene from *Streptococcus lactis* subsp. *diacetylactis* in *Escherichia coli*. Appl. Environ. Microbiol. **54**:1889–1891.
9. **Gottschalk, G.** 1979. Bacterial metabolism. Springer-Verlag, New York.
10. **Guardiola, J., M. DeFelice, and M. Iaccarino.** 1974. Mutant of *Escherichia coli* K-12 missing acetolactate activity. J. Bacteriol. **120**:536–538.
11. **Halpern, Y. S., and H. E. Umbarger.** 1959. Evidence for two distinct enzyme systems forming acetolactate in *Aerobacter aerogenes*. J. Biol. Chem. **234**:3067–3071.
12. **Harms, E., J.-H. Hsu, C. S. Subrahmanyam, and H. E. Umbarger.** 1985. Comparison of the regulatory regions of *ilvGEDA* operons from several enteric organisms. J. Bacteriol. **164**:207–216.
13. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
14. **Hohn, B., and K. Murray.** 1977. Packaging recombinant DNA molecules into bacteriophage particles *in vitro*. Proc. Natl. Acad. Sci. USA **74**:3259–3263.
15. **Irie, S., S. Doi, T. Yorifuji, M. Takagi, and K. Yano.** 1987. Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*. J. Bacteriol. **169**:5174–5179.
16. **Johansen, L., K. Bryn, and F. C. Størmer.** 1975. Physical and biochemical role of the butanediol pathway in *Aerobacter* (*Enterobacter*) *aerogenes*. J. Bacteriol. **123**:1124–1130.
17. **Juni, E.** 1952. Mechanisms of formation of acetoin by bacteria. J. Biol. Chem. **195**:715–726.
18. **Krook, M., L. Marekov, and H. Jörnvall.** 1990. Purification and structural characterization of placental NAD^+-linked 15-hydroxyprostaglandin dehydrogenase. The primary structure reveals the enzyme to belong to the short-chain alcohol dehydrogenase family. Biochemistry **29**:738–743.
19. **Lawther, R. P., D. H. Calhoun, C. W. Adams, C. A. Hauser, J. Gray, and G. W. Hatfield.** 1981. Molecular basis of valine resistance in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **78**:922–925.
20. **Løken, J. P., and F. C. Størmer.** 1970. Acetolactate decarboxylase from *Aerobacter aerogenes*. Purification and properties. Eur. J. Biochem. **14**:133–137.
21. **Loviny, T., P. M. Norton, and B. S. Hartley.** 1985. Ribitol dehydrogenase of *Klebsiella aerogenes*: sequence of the structural gene. Biochem. J. **230**:579–585.
22. **Magee, R. J., and N. Kosaric.** 1987. The microbial production of 2,3-butanediol. Adv. Appl. Microbiol. **32**:89–161.
23. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835.
24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mizusawa, S., S. Nishimura, and F. Seela.** 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14**:1319–1324.
26. **Navre, M., and G. M. Ringold.** 1988. A growth factor-repressible gene associated with protein kinase C-mediated inhibition of adipocyte differentiation. J. Cell Biol. **107**:279–286.
27. **Ohshiro, T., A. Kazuo, and T. Uwajima.** 1989. Purification and characterization of α-acetolactate decarboxylase from *Brevibacterium acetylicum*. Agric. Biol. Chem. **53**:1913–1918.

27a. **Olsen, F., and K. Aunstrup.** December 1984. Denmark patent EP 0 128 714.

28. **Penttilä, M. E., K. M. H. Nevalainen, A. Raynal, and J. K. C. Knowles.** 1984. Cloning of *Aspergillus niger* genes in yeast. Expression of the gene coding *Aspergillus* β-glucosidase. Mol. Gen. Genet. **194**:494–499.
29. **Prescott, S. C., and C. G. Dunn.** 1959. The production and properties of 2,3-butanediol, p. 399–427. *In* C. G. Dunn (ed.), Industrial microbiology, 3rd ed. McGraw-Hill Book Company, Inc., New York.
30. **Raibaud, O., and M. Schwartz.** 1984. Positive control of transcription in bacteria. Annu. Rev. Genet. **18**:173–206.
31. **Rasmussen, A. M., R. M. Gibson, S. E. Godtfredsen, and M. Ottesen.** 1985. Purification of α-acetolactate decarboxylase from *Lactobacillus casei* DSM 2547. Carlsberg Res. Commun. **50**:73–82.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Shaw, K. J., C. M. Berg, and T. J. Sobol.** 1980. *Salmonella typhimurium* mutants defective in acetohydroxy acid synthase I and II. J. Bacteriol. **141**:1250–1263.
34. **So, M., H. Boyer, M. Betlach, and S. Fallow.** 1978. Molecular cloning of an *Escherichia coli* plasmid determinant that encodes for the production of heat-stable enterotoxin. J. Bacteriol. **128**:463.
35. **Sone, H., T. Fujii, K. Kondo, F. Shimizu, J.-I. Tanaka, and T. Inoue.** 1988. Nucleotide sequence and expression of the *Enterobacter aerogenes* α-acetolactate decarboxylase gene in brewer's yeast. Appl. Environ. Microbiol. **54**:38–42.
36. **Spiro, S., and J. R. Guest.** 1990. FNR and its role in oxygen-regulated gene expression in Escherichia coli. FEMS Microbiol. Rev. **75**:399–428.
37. **Squires, C. H., M. DeFelice, J. Devereux, and J. M. Calvo.** 1983. Molecular structure of *ilvIH* and its evolutionary relationship to *ilvG* in *Escherichia coli* K-12. Nucleic Acids Res. **11**:5299–5313.
38. **Stahl, U., and D. Goelling.** 1990. Isolation and expression of the α-acetolactate decarboxylase gene of *Acetobacter pasteurianus*. European Brewery Convention Bulletin, Microbiology Group Technical Meeting, Copenhagen, 1990, p. 83–94.
39. **Størmer, F. C.** 1968. Evidence for induction of the 2,3-butanediol-forming enzymes in *Aerobacter aerogenes*. FEBS Lett. **2**:36–38.
40. **Størmer, F. C.** 1975. 2,3-Butanediol biosynthetic system in *Aerobacter aerogenes*. Methods Enzymol. **41**:518–533.
41. **Suihko, M.-L., K. Blomqvist, M. Penttilä, R. Gisler, and J. K. C. Knowles.** 1990. Recombinant brewer's yeast strains suitable for accelerated brewing. J. Biotechnol. **14**:285–300.
42. **Suihko, M.-L., M. Penttilä, H. Sone, S. Home, K. Blomqvist, J. Tanaka, T. Inoue, and J. K. C. Knowles.** 1989. Pilot brewing with α-acetolactate decarboxylase active yeasts, p. 483–490. *In* European Brewery Convention, Proceedings of the 22nd Congress, Zürich, 20–25 May 1989. IRL Press, Oxford University Press, Oxford.
43. **Summers, W. C.** 1970. A simple method for extraction of RNA

from *E. coli* utilizing diethylpyrocarbonate. Anal. Biochem. **33**:459–463.
44. **Umbarger, H. E.** 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. **47**:533–606.
45. **Voges, O., and B. Proskauer.** 1898. Beträg zur Ernährungsphysiologie und der Differentialdiagnose der Bakterien der hämorrhagischen Septicamie. Z. Hyg. Infektionskr. **28**:20–22.
46. **Wek, R. C., C. A. Hauser, and G. W. Hatfield.** 1985. The nucleotide sequence of the *ilvBN* operon of *Escherichia coli*: sequence homologies of the acetohydroxy acid synthase isozymes. Nucleic Acids Res. **13**:3995–4010.
47. **Zagursky, R. J., M. L. Berman, K. Baumeister, and N. Lomax.** 1986. Rapid and easy sequencing of large linear double stranded DNA and supercoiled plasmid DNA. Gene Anal. Technol. **2**:89–94.