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Cloning and Nucleotide Sequence of the *Lactobacillus casei* Lactate Dehydrogenase Gene

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An allosteric L-(+)-lactate dehydrogenase gene of *Lactobacillus casei* ATCC 393 was cloned in *Escherichia coli*, and the nucleotide sequence of the gene was determined. The gene was composed of an open reading frame of 981 bp, starting with a GTG codon and ending with a TAA codon. The sequences for the promoter and ribosome binding site were identified, and a sequence for a structure resembling a ρ-independent transcription terminator was also found.

Allosteric L-(+)-lactate dehydrogenase (LDH; EC 1.1.1.27) regulated by fructose-1,6-diphosphate (FDP) is a key enzyme in lactic acid fermentation by most group N streptococci and lactobacilli. Strains of *Streptococcus lactis* produce lactate as the only end product when grown on glucose or lactose, whereas they additionally produce formate, acetate, and ethanol from pyruvate when grown on galactose or limited concentrations of glucose. This production is correlated with lower levels of allosteric LDH and its activator, FDP (29, 30). Metabolic pathways of homofermentative *Lactobacillus casei* are controlled by the availability and kinds of carbohydrates, which determine the level of FDP and triosephosphate metabolic intermediates and subsequently control the activity of allosteric LDH and other enzymes to produce fermentative end products other than lactic acid. Allosteric regulation by FDP has been investigated (7, 13–15, 17). Recently, FDP-independent control of LDH was reported in *L. bulgaricus* (23). When this organism is grown in continuous cultures with limiting glucose, a shift in the pH of the medium from the acidic to the alkaline range causes this normally homofermentative bacterium to catabolize glucose in a heterofermentative fashion through reduced synthesis of LDH together with participation of the phosphoroclastic split pathway. These findings imply that the LDHs in lactic acid bacteria are under the control of not only allosteric effects but also the regulation of gene expression. To initiate studies of controlled gene expression, we have cloned a gene (*ldh*) encoding allosteric LDH of *L. casei* ATCC 393 and investigated its primary structure.

Standard recombinant DNA techniques (25) were used. Gene donor *L. casei* cells were grown at 37°C to the early stationary phase in MRS broth (Difco Laboratories) containing 20 mM DL-threonine to facilitate cell lysis, as described by Chassy et al. (3). The cells were harvested and lysed, and chromosomal DNA was isolated by the procedure described for *Bacillus subtilis* chromosomal DNA preparations (24). The chromosomal DNA was partially digested with *Alu*I, and fragments ranging from 0.5 to 6 kb were ligated with *Sma*I-digested and dephosphorylated pUC19 (34) by use of T4 DNA ligase. The resulting ligation mixture was used to transform *Escherichia coli* JM83 (31) by the competency method described previously (11). Approximately 20,000 colonies of *E. coli* transformants were obtained, and 5,000 of them were subjected to colony hybridization by the procedure of Grunstein and Hogness (10). The previously determined amino acid sequence (15) was used to design oligonucleotide mixtures as hybridization probes. Sequences for the mixtures were GAC/T AAA/G GAC/T CAC/T CAA/G AAA/G GT, ATG GGA/G/C/T GAA/G CAC/T GG, and ATG GAC/T GGA/G/C/T CAA/G TAC/T GG, representing all possible coding sequences of amino acid residues 17 to 24, 192 to 196, and 275 to 280, respectively. Hybridization and washing temperatures were determined by the formulas (32) 2°C per A/T base pair and 4°C per G/C base pair, with a range from 42 to 62°C for all of the synthetic probes. A series of dot blot experiments revealed that 45°C was optimal. After colony hybridization, whole-cell crude extracts were prepared from cultures that were inoculated with 16 colonies that showed hybridization signals and were assayed to determine their LDH levels. Allosteric LDH activity was determined as described by Hensel et al. (13), with some modifications. The 2-ml (total-volume) reaction mixture contained 17 mM pyruvate, 0.165 mM NADH, 3 mM FDP, 10 mM $MnSO_4$, and 50 mM acetate buffer (pH 5.4). One unit of allosteric LDH was taken as the amount of enzyme required to catalyze the oxidation of 1 μmol of NADH per min. Finally, only one clone was found to carry the *ldh* gene.

A recombinant plasmid was isolated from the clone showing allosteric LDH activity and designated pLS65. A restriction map of pLS65 was constructed (data not shown). The chromosomal insert of pLS65 was fragmented with restriction enzymes and cloned in *E. coli* JM83 with pUC19. Both strands of the insert were sequenced by the dideoxynucleotide method of Sanger et al. (26) with Sequenase version 2.0 (United States Biochemical). A 1,451-bp sequence was determined, and an amino acid sequence was deduced from the coding region (Fig. 1).

S1 nuclease mapping was performed by a previously described method (25). Total RNA was isolated from *E. coli* JM83 carrying pLS65 as described by Ausubel et al. (1), and a 460-bp fragment of pLS65 containing the promoter was used as a probe. After S1 nuclease digestion, the S1 nuclease-resistant hybrids were precipitated with ethanol and analyzed by electrophoresis in 6% polyacrylamide gels containing 7 M urea and autoradiography (Fig. 2).

The presumed ribosome binding sequence (Fig. 1) was confirmed by deleting a 12-bp region containing this sequence. An *Sph*I site upstream from the putative ribosome

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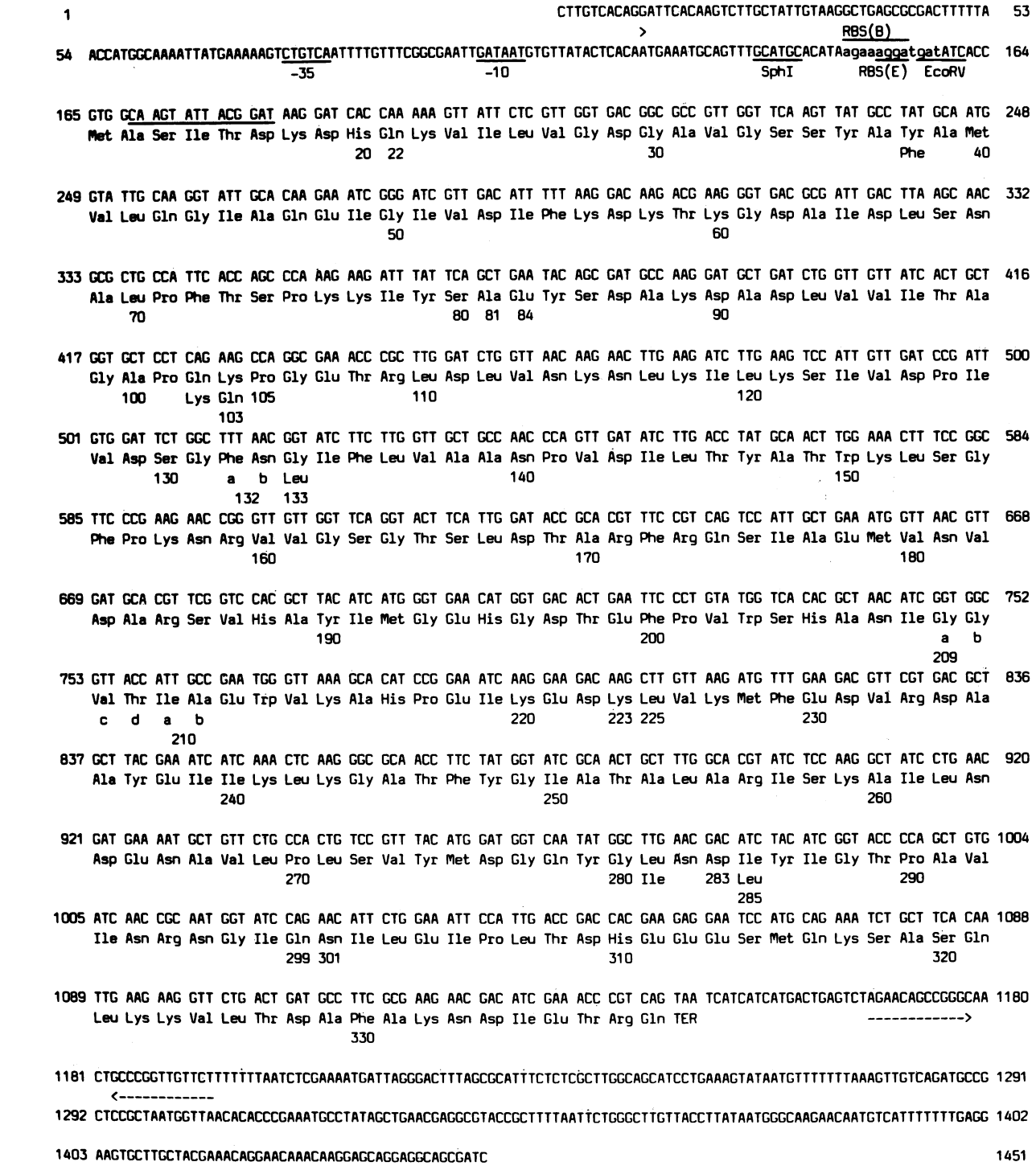

FIG. 1. Nucleotide sequence of the *L. casei ldh* gene and deduced amino acid sequence. The amino acid residues are numbered as described by Eventoff et al. (8). Promoter sequences homologous to the −35 and −10 regions of the *E. coli* σ^{70} or *B. subtilis* σ^{43} recognition sequences are underlined and labeled −35 and −10, respectively. The transcription start site, determined by S1 nuclease mapping, is denoted by > above the site. The deleted sequence used to confirm the putative ribosome binding site (see the text) is indicated by lowercase letters, and the ribosome binding sequences identical to those of *E. coli* and *B. subtilis* are indicated by RBS(E) and RBS(B) below and above the sequences, respectively. Following the start codon, a sequence homologous to the downstream box (28) is underlined. Discrepancies between the previously determined amino acid sequence (15) and our sequence are shown at their corresponding positions. An inverted repeat capable of forming a stem-loop structure is marked by horizontal arrows.

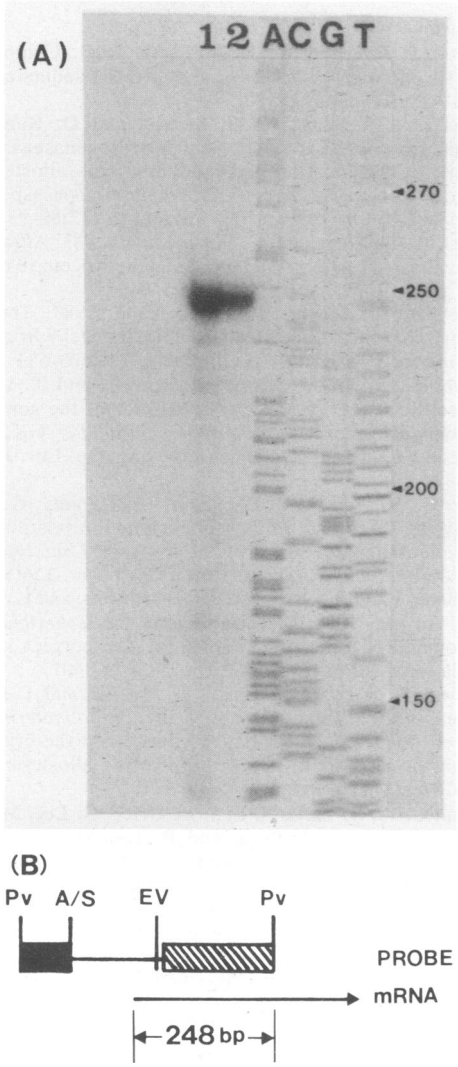

FIG. 2. S1 nuclease mapping for identification of the transcription initiation site of the *L. casei ldh* gene. (A) Lanes: 1 and 2, S1 nuclease mapping performed with independently prepared RNAs. S1 nuclease-resistant *Pvu*II fragment of pLS65 hybridized to RNA independently isolated from *E. coli* carrying pLS65. Lanes A, C, G, and T, dideoxy nucleotide sequence ladder of M13mp18 as a size marker. The numbers in the margin represent the lengths of fragments in nucleotides. (B) Schematic representation of mRNA and the promoter-containing *Pvu*II fragment of pLS65 used as a probe. The length of the fragment protected by mRNA was 248 nucleotides. Symbols: ■, pUC19; ——, 5′-untranslated region of the *ldh* gene; ▨, translated region of the *ldh* gene; Pv, *Pvu*II; EV, *Eco*RV; A/S, ligation site for *Alu*I and *Sma*I ends.

binding site was used in a fusion with pUC19. The *Hin*dIII site of pUC19 following the fusion site of *Sph*I was cut, and the Klenow fragment was used for filling in to generate a blunt end. Another blunt end downstream from the putative ribosome binding site was generated by *Eco*RV digestion, and both blunt ends were ligated, giving rise to a deletion of 12 bp, shown by lowercase letters in Fig. 1. The resultant plasmid was designated pLS65D.

As predicted, the entire *ldh* gene was shown to lie within the 1.65-kb insert of pLS65 (Fig. 1). The open reading frame of 981 bp started with a GTG initiation codon and ended with a TAA stop codon. The deduced amino acid sequence agreed with the previously determined sequence (15), except at six positions. Amino acid residues 38, 133, 281, and 285 were replaced by Tyr, Gly, Leu, and Ile, respectively. These exchanges are outside the conserved residues involved in catalysis, allosteric regulation, and recognition for substrate and coenzyme (4, 5). However, residues Lys-102 and Gln-103 of the previously determined sequence (15) were inverted to Gln-102 and Lys-103 in our sequence, and Gln-102 has been known to be highly conserved and important in substrate discrimination (6, 33). The complete amino acid sequence deduced from the nucleotide sequence of our *L. casei ldh* gene was compared with the amino acid sequences of LDHs from *Bifidobacterium longum* (19), *Bacillus stearothermophilus* (2), *Thermus caldophilus* (16), and *T. aquaticus* (21). The amino acid sequence homology among these bacterial LDHs was not higher than 38%. However, all of the amino acid residues of the LDHs involved in substrate recognition (Asp-197, Thr-246, and Gln-102), catalysis (Arg-109, Arg-171, Ile-250, His-195, and Asp-168), and allosteric regulation (Arg-173 and His-188), as reviewed by Clarke et al. (4, 5), were found to be perfectly conserved.

The transcription initiation site determined by S1 nuclease mapping (Fig. 2) was found to be an A 43 bp upstream from the initiation codon; accordingly, a pair of sequences, CTG TCA and GATAAT (Fig. 1) were assumed to be −35 and −10 regions, respectively, with a spacing of 18 bp; this arrangement is similar to the consensus sequence for *E. coli* promoters (12) or to those for promoters of gram-positive organisms (9, 20). An additional conserved region with a cluster of A residues, as compiled by Graves and Rabinowitz (9), was also found and presumed to be a promoter element of gram-positive bacteria.

In the region 10 bp upstream from the start codon (Fig. 1), we found the sequences AGGA and AGAAAGGA, which are known to be ribosome binding sites for *E. coli* (27) and *B. subtilis* (20), respectively. When this region was deleted, no translation product of the *ldh* gene was identified on a sodium dodecyl sulfate-polyacrylamide gel, and no allosteric LDH activity was detected in the whole-cell crude extract of *E. coli* JM83 carrying pLS65D (data not shown). These results are contrary to the suggestion by Melancon et al. (18) that the Shine-Dalgarno sequence in *E. coli* is not essential for the correct selection of translational start sites. Following the start codon, a 14-bp sequence (Fig. 1) which was homologous to the downstream box proposed by Sprengart et al. (28) was found. Sprengart et al. suggest that the mRNA sequence transcribed from the downstream box also plays a role in translation initiation by base pairing with the anti-downstream box defined in 16S rRNA of *E. coli*.

Downstream from the TAA stop codon, we found a sequence for a structure resembling the *E. coli* ρ-independent transcription terminator. The RNA transcribed from this region would form a stem-loop structure with a GC-rich sequence followed by a stretch of U residues (Fig. 1). The calculated free energy of formation for this structure would be −19.4 kcal (ca. −81.2 kJ), within the range typically observed for ρ-independent terminators (22).

Aliquots from each culture of *E. coli* JM83 harboring pLS65 and *L. case* ATCC 393 were collected to estimate enzyme production during growth (Fig. 3). Both organisms produced about the same maximum amount of allosteric LDH, i.e., 13 U/mg of protein. However, the modes of enzyme production by the two organisms during growth were different. *E. coli*(pLS65) produced allosteric LDH consistently throughout the growth phase, typical for *E. coli* promoters recognized by σ^{70} (12), while enzyme production

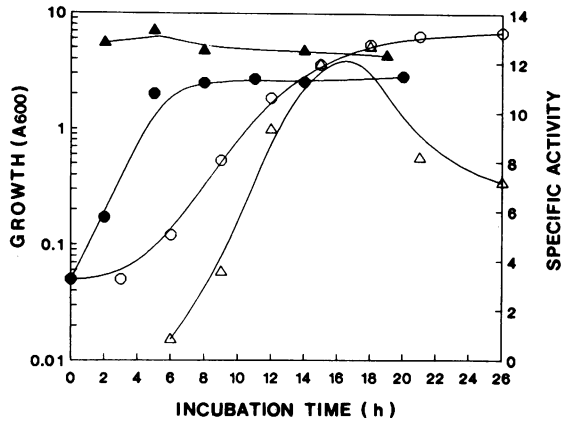

FIG. 3. Growth and LDH production of *E. coli*(pLS65) and *L. casei*. The organisms were grown separately, and their growth was measured by reading the A_{600}. The allosteric LDH assay was carried out as described in the text. Symbols: ●, growth of *E. coli* carrying pLS65; ○, growth of *L. casei*; ▲, specific activity of allosteric LDH in *E. coli*; △, specific activity of allosteric LDH in *L. casei*.

by *L. casei* was growth associated, typical of *B. subtilis* promoters recognized by σ^{43} (9, 20).

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