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Cloning of the D-lactate dehydrogenase gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* by complementation in *Escherichia coli*

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A strain of *Escherichia coli* (FMJ144) deficient for pyruvate formate lyase and lactate dehydrogenase (LDH) was complemented with a genomic DNA library from *Lactobacillus delbrueckii* subsp. *bulgaricus*. One positive clone showed LDH activity and production of D(−)lactate was demonstrated. The nucleotide sequence of the D-LDH gene (*ldhA*) revealed the spontaneous insertion of an *E. coli* insertion sequence IS*2* upstream of the gene coding region. The open reading frame encoded a 333-amino acid protein, showing no similarity with known L-LDH sequences but closely related to *L. casei* D-hydroxyisocaproate dehydrogenase (D-HicDH).

D-Lactate dehydrogenase; Insertion sequence IS2; *Lactobacillus delbrueckii* subsp. *bulgaricus*; D-Hydroxyisocaproate dehydrogenase

1. INTRODUCTION

The NADH-dependent lactate dehydrogenase (LDH) is a key enzyme in the fermentative metabolism of lactic acid bacteria, since it allows re-oxidation of the NAD needed for glycolysis through the coupled reduction of pyruvate to lactate. Two configurations are known for lactate: L(+) and D(−). Stereospecificity is achieved by distinct enzymes called L-LDH and D-LDH. Lactic acid bacteria produce either one or the two forms of lactate. For example, more than 90% of the lactate produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* (in short, *L. bulgaricus*) is of the D-form [1].

Little is known about the evolutionary relationship between L- and D-LDH. More than 25 years ago, the suggestion was made that these two enzymes had a common ancestry [2]. This claim was based upon limited peptide analysis and remained unchallenged, due to the lack of relevant information about D-LDH as opposed to L-LDH of which the primary and tertiary structures are known in detail [3]. Two recent reports provide some insights into this fundamental problem. Le Bras and Garel [4] and Clarke et al. [5] just reported the sequence of about 50 N-terminal amino acids of *L. bulgaricus* D-LDH. This sequence bears no resemblance to L-LDH but, as Clarke et al. [5] pointed out, closely matches the corresponding region of *L. casei* D-hydroxyisocaproate dehydrogenase (D-HicDH) [6]. Thus, the opposite view that D- and L-LDH are descended from distinct evolutionary ancestors is now favoured.

In this paper, we report for the first time the complete genomic sequence of a D-LDH gene cloned from *L. bulgaricus*. We show that this protein exhibits no similarity with the L-LDH framework besides the co-enzyme binding site, but that it is closely related to *L. casei* D-HicDH.

Abbreviations: L-LDH, L-lactate dehydrogenase (EC 1.1.1.27); D-LDH, D-lactate dehydrogenase (EC 1.1.1.28); D-HicDH, D-hydroxyisocaproate dehydrogenase.

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2. MATERIALS AND METHODS

General molecular biology and culture techniques were performed according to the instructions and recipes given by Sambrook et al. [7].

2.1. *Bacterial strains and plasmids*

Lactobacillus delbrueckii subsp. *bulgaricus* LMG6901 (= NCIB 11778) was obtained from Dr D. Janssens (LMG Culture Collection, Lab. voor Microbiologie, Gent). *E. coli* FMJ 144 (pfl^- ldh^- Cm^R) was obtained from David P. Clark, Southern Illinois University, Carbondale [8]. The vectors used were pJDC9 [9] for complementation and pBluescript phagemids (Stratagene) for subcloning and sequencing.

2.2. *Growth and selection media*

L. bulgaricus was grown in 25-ml bottles at 37°C without shaking in MRS broth (Difco 0881). For complementation, FMJ144 strain was grown anaerobically at 37°C on M9 minimal plates with glucose 0.4% and amino acids 0.2%. The selected clones were grown aerobically at 37°C in LB broth. Antibiotics were used at the following concentrations (in μg/ml): ampicillin 100, chloramphenicol 50, erythromycin 250.

2.3. *DNA preparation and construction of the genomic library*

L. bulgaricus was grown to mid-log phase in 600 ml of MRS medium. Cells were pelleted and resuspended in 4 ml TEN buffer (Tris-

HCl 100 mM, EDTA 100 mM, NaCl 150 mM, pH 8). Lysozyme (20 mg/ml) and mutanolysin (Sigma; 50 μg/ml) were added. Lysis was performed successively at 4°C for 2 h, at −20°C for 25 min and finally at 95°C for 10 min. After RNase A addition (50 μg/ml), incubation was continued at 50°C for 15 min and then at 65°C for 1 h after addition of proteinase K (100 μg/ml) and SDS (1%). The lysate was extracted 3 times with a mixture of chloroform and iso-amyl alcohol (24 vol/1 vol). The aqueous phase was removed and placed in a 50-ml glass cylinder. DNA was precipitated with the addition of ice-cold absolute ethanol and then rolled around sterile glass sticks. The DNA was dried and dissolved in 1 ml Tris-HCl 10 mM, EDTA 1 mM, NaCl 50 mM, pH 8. Chromosomal DNA was totally digested with *Bam*HI, cloned in pJDC9 vector and transferred into FMJ144 strain by electroporation.

2.4. *Selection by complementation*

Transformed cells were plated onto M9 medium and incubated anaerobically at 37°C. Colonies growing after a few days were isolated on M9 with chloramphenicol and erythromycin. Transformants were further analysed by plasmid isolation and restriction, back transformation and enzyme activity assays.

2.5. *Enzyme assays*

LDH activity was assayed with pyruvate on crude cell extracts. The assay mixture (1 ml) contained phosphate buffer at pH 5.6 (KH_2PO_4 73 mM, Na_2HPO_4 3.5 mM), NADH 0.2 mM and 1 to 50 μl crude extract. Sodium pyruvate 1 mM was added to start the reaction. NADH oxidation was followed by the decrease in absorbance at 340 nm.

2.6. *Lactic acid assay*

Production of lactate in the culture medium was measured spectrophotometrically. Lactate was oxidised by commercial stereospecific LDH in the presence of NAD. The increase in absorbance at 340 nm was used to calculate the concentration of L(+) and D(−) lactate in the supernatants of 16 h anaerobic cultures (Boehringer Mannheim, kit 1 112 821).

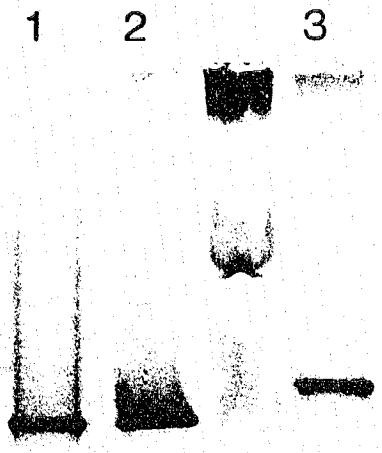

Fig. 1. Non-denaturating PAGE of LDH stained for activity on D/L lactate. Lane 1, crude extracts from *L. bulgaricus*; lane 2, crude extract from *E. coli* FMJ144(pGIN012); lane 3, purified D-LDH from *L. leichmanii* (Boehringer Mannheim). A similar analysis performed on L-lactate gave no activity for the three enzymes tested.

2.7. *PAGE analysis*

Crude cell extracts from *L. bulgaricus* and from the *E. coli* transformants were analysed by PAGE analysis (10% acrylamide) under non-denaturating conditions (Tris 250 mM, Glycin 200 mM, pH 8.3). Gels were stained with Nitroblue tetrazolium (0.2 mg/ml) in the presence of NAD (0.5 mg/ml) with either L-lactate (4.8 mg/ml) or a mixture of L- and D-lactate (1/1, 9.6 mg/ml).

2.8. *DNA sequence*

Sequencing reactions were carried out on single-strand DNA by the primer walking technique using Sequenase (USB). Computer analysis was performed using PC/GENE (Intelligenetics).

3. RESULTS AND DISCUSSION

3.1. *Cloning of the* L. bulgaricus *D-LDH gene*

FMJ144 strain was electroporated with a ligation mixture containing approximately 200 000 recombinant plasmids and transformants complemented for growth in the absence of oxygen were selected on M9 minimal medium. 60 positive colonies were streaked on LB plates with erythromycin and chloramphenicol and incubated anaerobically at 37°C. Twelve colonies growing in the presence of the antibiotics were retained for further analysis. They were assayed for LDH activity together with *L. bulgaricus* and *E. coli* FMJ144 (pJDC9) as controls. One clone harboring a plasmid with a 3.4 kb *Bam*HI insert (named pGIN012) was shown to actively produce D-LDH. Lactate was shown to accumulate in the culture medium at a concentration of 0.55 g/l after 16 h of anaerobic culture. No lactate could be

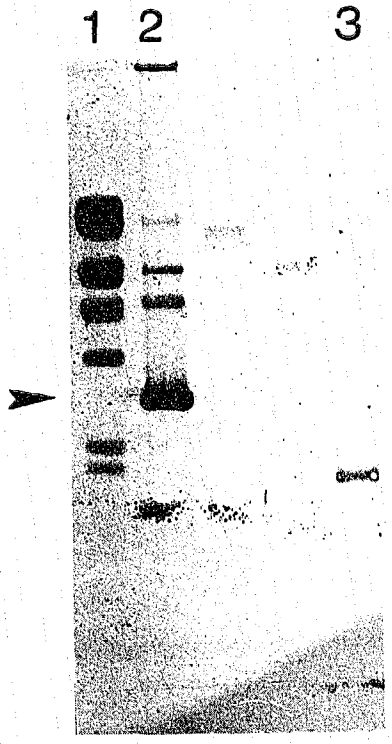

Fig. 2. Southern blot analysis. Lane 1, *Hin*dIII-restricted phage λ; lane 2, *Bam*HI-restricted pGIN012; lane 3, *Bam*HI-restricted *L. bulgaricus* genomic DNA. The probe is the *Bam*HI insert (arrow) of pGIN012 (upper bands in lane 2 are partial digestion products.).

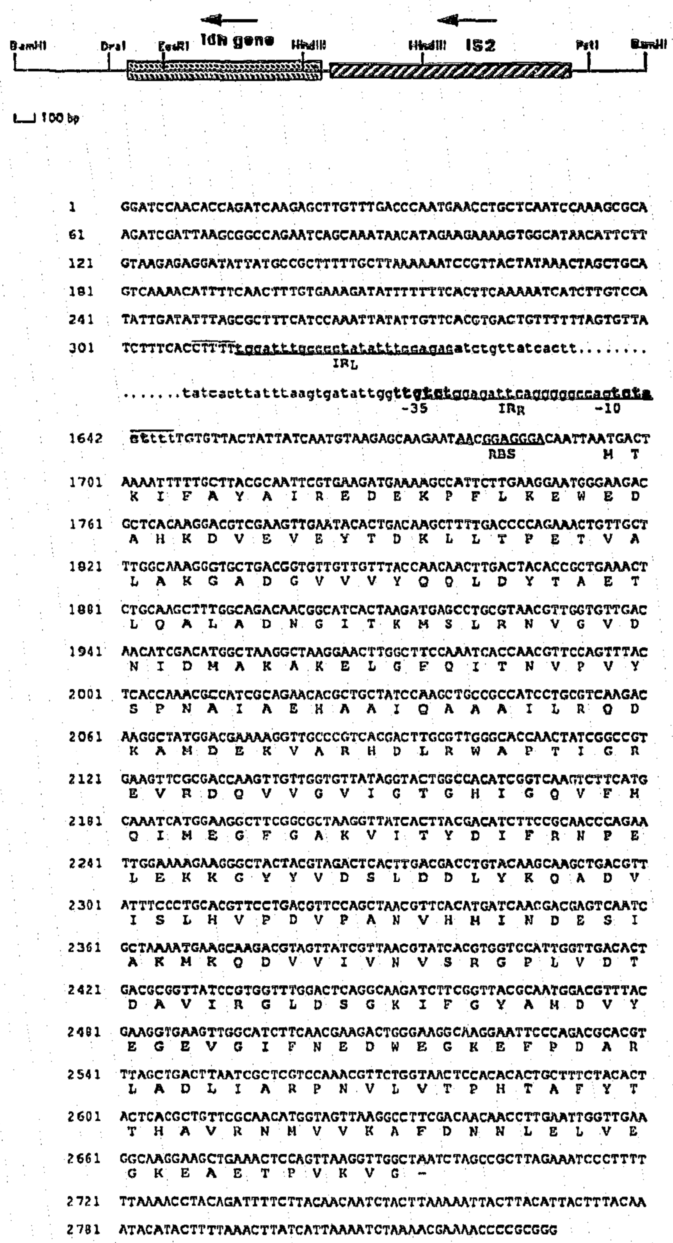

Fig. 3. Nucleotide sequence and deduced amino acid sequence of D-LDH. The structure of the pGIN012 insert is outlined at the top. The sequence starts at the right *Bam*HI cloning site. Only part of the IS*2* sequence is given (lower case letters; underlined: inverted terminal repeats; overlined: target site direct repeats). The −35 and −10 boxes of a a putative substitute promoter brought by IS*2* are outlined. The presumed ribosome binding site (RBS) is underlined.

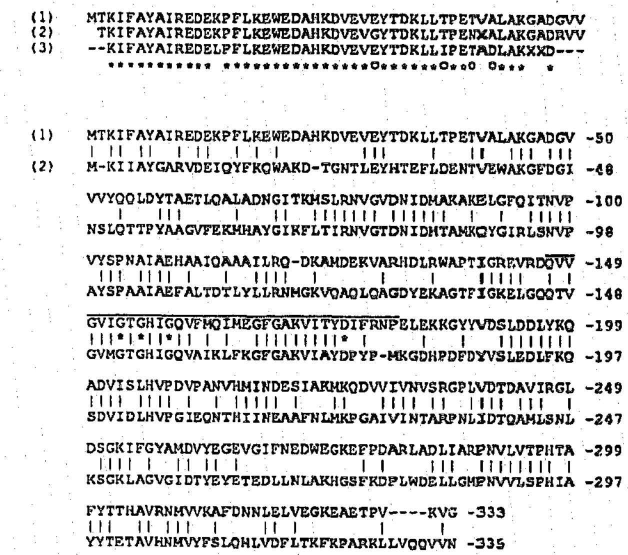

Fig. 4. Upper panel: comparisons (CLUSTAL) between the N-terminal sequences of *L. bulgaricus* D-LDH (line 1, this work; line 2, ref. [4]; line 3, ref [5]; (*), identical amino acid; (○, similar amino acid). Lower panel: comparisons (PALIGN) between *L. bulgaricus* D-LDH (line 1, this work) and *L. casei* D-HicDH (line 2, ref. [6]). The NADH-binding site [6] is overlined; the totally conserved residues of the motif [13] are marked by a star.

detected in the FMJ144 (pJDC9) supernatant. The lactate produced was exclusively of the D(−) type. PAGE analysis (Fig. 1) confirmed the stereospecificity in the cloned enzyme and showed that the enzymes produced in *L. bulgaricus* and in *E. coli* (pGIN012) had identical electrophoretic mobilities. A partial restriction map of pGIN012 was established (Fig. 3). The 3.4-kb insert was digested with *Bam*HI and *Eco*RI and subcloned in pBluescript vectors. The resulting subclones, which were defective in complementation, were used for sequencing.

A Southern blot analysis was performed on *L. bulgaricus* chromosomal DNA using as a probe the 3.4-kb *Bam*HI insert from pGIN012 containing the D-LDH gene. Fig. 2 shows that the cloned *Bam*HI fragment was larger (by about 1.8 kb) than its chromosomal homologue. According to the restriction map and in view of other examples in the literature [10,11], we suspected the insertion of an *E. coli* insertion sequence, IS*2*. This was confirmed by sequence analysis (see below).

3.2. *Sequence analysis*

The sequence of the *L. bulgaricus* D-LDH gene (*ldhA*) is presented in Fig. 3. Its open reading frame is preceded by a putative ribosome-binding site and encodes a 333-amino acid protein with a predicted molecular weight of 37 079 Da.

Insertion of the mobile element IS*2* was confirmed by the sequence. This insertion occurred just upstream of the gene coding region. We believe it was beneficial for the cloning of the D-LDH gene in *E. coli* due to the disruption of the genuine structure of the locus likely to be detrimental or lethal for this host. This view is based upon the reported difficulties encountered during the cloning of the closely related D-HicDH gene [6] and is further strengthened by our observation that the pGIN012 clone exhibits very poor growth. A positive effect for IS*2* insertion might be the subtraction of the D-LDH coding region from the influence of its own promoter, which might be too strong. This implies that IS*2* should provide a substitute, weaker promoter. In

agreement with this suggestion, inspection of the IS*2* sequence proximal to the D-LDH coding region reveals the presence of a promoter-like sequence. As to the genuine D-LDH promoter, several candidates (about 10) can be identified within a 100-nucleotide stretch preceding the ribosome-binding site, some of them being flanked by TG dinucleotides typical of Gram+ promoters [12]. We speculate that this segment of DNA, bearing multiple consensual promoters, is likely to drive D-LDH expression in *E. coli* to a lethal level.

The deduced N-terminal protein sequence closely matches (Fig. 4, upper panel) the corresponding reported segments of *L. bulgaricus* D-LDH enzymes [4,5] except for 6 substitutions (4 conservative) which most probably result from strain polymorphism. The D-LDH framework is highly similar (53%) to *L. casei* D-HicDH [6] (Fig. 4, lower panel), in agreement with the conclusion recently drawn by Clarke et al. [5] from limited N-terminal sequence comparison. No similarity could be found with L-LDH, besides the NADH-binding motif [6] common to all NADH-dependent dehydrogenases (in particular the GxGxxG adenine ribose motif and the 'D53' aspartate which selects NAD from NADP; ref. [13]), nor with any other protein sequence available in data banks. Our results therefore definitely demonstrate that D-LDH pertains to a family of NADH-dependent dehydrogenases distinct from L-LDH, as suggested by Clarke et al. [5]. Both enzymes catalyse the same redox reaction and they share a common motif for the binding of co-enzyme. Yet, their overall framework is different. The molecular architecture of L-LDH is known with high precision and representatives of the family, although widely spread among bacteria as well as higher organisms, strictly fit a common design [3]. The cloning of D-LDH and the demonstration of its close similarity to D-HicDH have revealed the existence of a new family of enzymes devoted to the same redox function but with a different structure.

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