Cloning, sequencing and comparison of three lactococcal L-lactate dehydrogenase genes

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The conversion of pyruvate to lactate is a key feature of lactococcal strains. The enzyme which facilitates this conversion, L-lactate dehydrogenase (LDH), and the gene which encodes it (*ldh*), are therefore of great significance. This paper presents the cloning and DNA sequence analysis of three further lactococcal genes which are of key importance in the genetic manipulation of commercial starter strains. The *ldh* gene from *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* BU2-60 has been isolated from a lambda library and sequenced. The *ldh* gene from *L. lactis* subsp. *cremoris* NCDO 762 and that from *L. lactis* subsp. *lactis* IL1403 have been amplified by the polymerase chain reaction (PCR) and sequenced. These DNA sequences and deduced amino acid sequences have been compared with those from *L. lactis* subsp. *lactis* MG1363. The LDHs from *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* NCDO 762 are 99.4% homologous. The LDHs from *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *lactis* IL1403 are 96.4% homologous. The LDHs from *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *lactis* biovar *diacetylactis* BU2-60 are 99.9% homologous. Our results provide further evidence that *L. lactis* subsp. *lactis* MG1363 and other *L. lactis* subsp. *lactis* NCDO 712 derived strains should be reclassified as *Lactococcus lactis* subsp. *cremoris*.

Keywords: lactate dehydrogenase gene, lactococci, sequencing, classification

INTRODUCTION

The use of lactic acid bacteria in dairy fermentations is a process which has been in use for centuries. The strains in these processes have been primarily selected for their phenotypic traits. The classification of these strains has also been based upon their phenotypes. However, the rapid development of our understanding of the genetics underlying these phenotypic traits indicates that the grouping of strains by these criteria has led to some misclassifications.

The classification of strains belonging to the lactococcal subspecies *Lactococcus lactis* subsp. *lactis* (*L. lactis*) and *L. lactis* subsp. *cremoris* (*L. cremoris*) has been the subject of several investigations. A number of studies have indicated that *L. lactis* MG1363 (Gasson, 1983), *L. lactis* ML3, *L. lactis* C2 and *L. lactis* NCDO 763, all derivatives of *L. lactis* NCDO 712, are more closely related to *L. cremoris* than to *L. lactis*. Jarvis & Jarvis (1981) used DNA homology to compare 45 strains of lactic streptococci. Three strains of *L. lactis*, including *L. lactis* ML3, showed

greater homology with *L. cremoris* than with *L. lactis*. Salama *et al.* (1991) used subspecies specific oligonucleotide rRNA probes to identify *L. cremoris* strains. These probes hybridized with chromosomal DNA from *L. lactis* C2 but not with DNA from other *L. lactis* strains. Godon *et al.* (1992) showed that, under high stringency conditions, hybridization between DNA from *L. lactis* strains and DNA from *L. cremoris* strains was not typically found. Godon *et al.* (1992) demonstrated the hybridization of gene probes from *L. lactis* NCDO 763 with chromosomal DNA from *L. cremoris* AM2. Conversely, the hybridization of the same gene probe with chromosomal DNA from *L. lactis* NCDO 2118 could only be demonstrated at low stringency.

The continued advances in sequencing technology have resulted in the accumulation of a large number of gene sequences. The degree of DNA homology between bacterial strains may now be determined at the nucleotide level by comparing specific DNA sequences. Such comparisons of genes from *L. lactis* and *L. cremoris* further indicate the misclassification of *L. lactis* NCDO 712 derived strains. The *recA* sequences from *L. lactis* ML3 and *L. cremoris* IL736 are 98% homologous whereas the

Abbreviation: LDH, L-lactate dehydrogenase.

Fig. 1. Comparison of *ldh* gene sequences of (1) *L. cremoris* NCDO 762 (GenBank accession number U02385), (2) *L. lactis* biovar *diacetylactis* BU2-60 (U02386), (3) *L. lactis* IL1403 (U04315) and (4) *L. lactis* MG1363 (Griffin *et al.*, 1992). The amino acid sequence derived from the DNA sequence of *L. lactis* MG1363 is given below the DNA sequences. The single amino acid change in the LDH enzyme of *L. cremoris* NCDO 762 at amino acid 213 is shaded. The full DNA sequence of *ldh* from *L. lactis* MG1363 is given. Only bases which do not concur with this sequence data are given for the remaining genes. '-' indicates consensus between the three sequences.

recA sequence from *L. lactis* ML3 shows only 92% homology with *L. lactis* IL1403 (Duwat *et al.*, 1992), indicating the possible misclassification of *L. lactis* ML3. The X-prolyl dipeptidyl aminopeptidase (X-PDAP) gene (*pepXP*) sequences from *L. lactis* NCDO 763 and *L. cremoris* P8-2-47 show 99% homology (Nardi *et al.*, 1991; Mayo *et al.*, 1991).

In this study the sequences of the gene (*ldh*) which encodes L-lactate dehydrogenase (LDH) in *L. lactis* MG1363 (Griffin *et al.*, 1992), *L. lactis* IL1403, *L. lactis* biovar *diacetylactis* BU2-60 and *L. cremoris* NCDO 762 are compared. LDH is responsible for the formation of lactate

from the glycolytic product pyruvate. The accumulation of lactic acid is a significant phenotype when considering lactic acid bacteria such as the lactococci. In most instances the formation of lactate is the main route by which pyruvate is eliminated from the cell. Lactococcal strains designated as *L. lactis* biovar *diacetylactis* are one notable exception. These strains are named as a result of their ability to metabolize citrate and to then produce acetoin from the excess pyruvate accumulated. The significance of this pathway lies in the intermediate diacetyl, which is an important flavour compound. The gene encoding LDH in *L. lactis* MG1363 (Griffin *et al.*, 1992) has previously been cloned and sequenced. The sequence data for the *ldh*

Fig. 2. An evolutionary tree of bacterial *ldh* genes based on the progressive alignment method of Feng & Doolittle (1987) and KITSCH from the phylogeny inference package of Felsenstein (1985). The branch lengths reflect the extent of divergence. The bacterial sequences compared are those in Fig. 1 together with those from *Streptococcus mutans* (Hillman *et al.*, 1990), *Lactobacillus casei* (Kim *et al.*, 1991), *Lactobacillus plantarum* (Taguchi & Ohta, 1991), *Bacillus stearothermophilus* (Barstow *et al.*, 1986), *Bacillus caldolyticus* (Zulli *et al.*, 1987), *Bacillus caldotenax* (Barstow *et al.*, 1987), *Bacillus psychrosaccharolyticus* (Vckovski *et al.*, 1990), *Bacillus megaterium* (Waldvogel *et al.*, 1987), *Mycoplasma hyopneumoniae* (Haldimann *et al.*, 1993) and *Bifidobacterium longum* (Minowa *et al.*, 1989).

gene of *L. lactis* IL1403, *L. lactis* biovar *diacetylactis* BU2-60 and *L. cremoris* NCDO 762 are presented here and the sequences of the three *ldh* genes are compared.

METHODS

Bacterial strains and plasmids. *L. lactis* biovar *diacetylactis* BU2-60 is a plasmid free derivative of *L. lactis* biovar *diacetylactis* BU2 (Neve *et al.*, 1984). *L. lactis* IL1403 is a plasmid free strain (Chopin *et al.*, 1984). *L. cremoris* NCDO 762 was previously obtained from the National Collection of Food Bacteria, AFRC Institute of Food Research, Reading, UK. Lactococcal strains were propagated at 30 °C in M17 medium (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) carbohydrate. *Escherichia coli* MC1022 (Casadaban & Cohen, 1980) cells were maintained at 37 °C in L-broth. After transformation, *E. coli* 'one-shot' cells from the TA cloning kit (Invitrogen) were maintained at 37 °C in SOC medium (Hanahan, 1985).

Molecular techniques. The cloned *ldh* gene from *L. lactis* biovar *diacetylactis* BU2-60 was obtained from a λ EMBL3 library. Lambda clones containing the *ldh* gene were identified by polymerase chain reaction (PCR) screening (Griffin *et al.*, 1993). Lambda clone DNA was isolated using a Qiagen lambda kit (no. 12523). PCRs were carried out using a Hybaid TR2 thermal cycler. Ligations were performed according to Sambrook *et al.* (1989). Transformation of *E. coli* competent cells was as described by Cohen *et al.* (1972). PCR fragments were cloned using the Invitrogen TA Cloning system. Plasmid DNA was isolated by alkaline lysis (Sambrook *et al.*, 1989) and purified by caesium chloride density gradient centrifugation. Sequencing was performed using an Applied Biosystems (ABI) PRISM Terminator cycle sequencing kit and data obtained from an ABI 373 sequencer. Oligonucleotide primers for sequencing and PCR were produced using an ABI 392 synthesizer.

RESULTS

The LDH clone from *L. lactis* biovar *diacetylactis* BU2-60 was identified by PCR screening of plaques from the library (Griffin *et al.*, 1993). DNA was isolated using a Qiagen lambda kit. Appropriate restriction fragments of the lambda clone were identified as described previously (Griffin *et al.*, 1992). Two adjacent *EcoRV* fragments,

2.4 kb and 0.8 kb, of the lambda DNA were subcloned into *SmaI* digested pUC18 DNA and transformed into *E. coli* MC1022. The constructs were designated pFI730 (2.4 kb insert) and pFI727 (0.8 kb insert) and the strains were designated FI7901 and FI7939 respectively. The DNA sequence of the *ldh* gene contained in these inserts was determined (Fig. 1).

Using the sequence data already obtained for *L. lactis* MG1363, oligonucleotide primers which would amplify the complete *ldh* gene from *L. cremoris* NCDO 762 and *L. lactis* IL1403 were designed. The PCR products generated by these primer pairs were cloned into pCRII using the TA Cloning system. DNA was isolated from three independently cloned PCR fragments from each amplification and the DNA sequence determined (Fig. 1).

A comparison between the available lactococcal DNA sequences revealed closer homology between *L. lactis* MG1363 and *L. cremoris* NCDO 762 (99.4%) than between *L. lactis* MG1363 and *L. lactis* biovar *diacetylactis* BU2-60 (96.7%) or between *L. lactis* MG1363 and *L. lactis* IL1403 (96.4%). There was also a high degree of similarity between *L. lactis* biovar *diacetylactis* BU2-60 and *L. lactis* IL1403 (99.9% homology).

DISCUSSION

The gene encoding LDH is highly conserved across a wide range of organisms (Griffin *et al.*, 1992). Consequently little divergence between the *ldh* genes from two strains as closely related as *L. lactis* MG1363 and *L. lactis* biovar *diacetylactis* BU2-60 was expected. Cloning the *ldh* gene from *L. lactis* biovar *diacetylactis* BU2-60 gave the first indication that there was a significant difference in sequence from those genes already examined. The *ldh* gene from *L. lactis* MG1363 had been cloned in a single *EcoRV* fragment. Attempting to isolate the gene from *L. lactis* biovar *diacetylactis* BU2-60 showed that it could not be cloned in a single *EcoRV* fragment. Comparison of the *ldh* gene sequences of *L. lactis* MG1363 and BU2-60 reveals 32 base pair differences (96.7% homology). The

substitution of the C at 378 bp in the *ldh* of *L. lactis* MG1363 with a T in the *ldh* of *L. lactis* biovar *diacetylactis* BU2-60 was responsible for creating the *EcoRV* site. All 32 base changes are silent, occurring in the third, wobble, base of the codon.

The conclusions of Godon *et al.* (1992), Salama *et al.*, (1991) and Jarvis & Jarvis (1981) suggested that the possible misclassification of *L. lactis* MG1363 might account for these differences. The genetic comparisons of the *recA* genes and *pepXP* genes of *L. lactis* and *L. cremoris* indicated that a similar comparison of the *ldh* genes might also be of use. In order to perform this comparison the *ldh* gene from *L. cremoris* NCDO 762 was amplified from chromosomal DNA and cloned. Multiple clones were made and sequenced. The consensus of these sequences was used to eliminate any errors introduced by the use of PCR. The sequence data derived from *L. cremoris* NCDO 762 showed only six base pair differences when compared with that of *L. lactis* MG1363 (99.4% homology). This greater level of sequence homology agrees with the previous findings and reinforces the argument that *L. lactis* MG1363 is more closely related to *L. cremoris* strains than to *L. lactis* strains. However, it is interesting to note that one of the very few sequence differences between *L. lactis* MG1363 and *L. cremoris* NCDO 762 does result in an amino acid difference: a glutamic acid at position 213 in *L. lactis* MG1363 and a lysine in that position in *L. cremoris* NCDO 762. Griffin *et al.* (1992) described areas of very high conservation within the amino acid sequence of several LDH enzymes. This amino acid change lies outside these areas and is unlikely to affect enzymic function.

In order to perform a complete comparison of lactococcal *ldh* sequences the gene from *L. lactis* IL1403 was also amplified by PCR. Multiple clones were isolated and sequenced. Comparison of the *ldh* gene from *L. lactis* IL1403 with that of *L. lactis* biovar *diacetylactis* BU2-60 reveals only a single base pair difference (99.9% homology). This base change is silent. This high degree of homology accords with the close relationship between *L. lactis* biovar *diacetylactis* and true *L. lactis* strains.

The evolutionary tree in Fig. 2 depicts the phylogenetic relationships between the various bacterial *ldh* sequences available. The branch lengths reflect the extent of divergence. It is clear from this that the *L. lactis* biovar *diacetylactis* BU2-60 and *L. lactis* IL1403 genes emerged earlier than the divergence between the *L. lactis* MG1363 and *L. cremoris* NCDO 762 genes.

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