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

Simon R. Swindell, Hugh G. Griffin and Michael J. Gasson

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 subsp. lactis, 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
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.

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...
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 Institute of Food Research, Reading, UK. Lactococcal strains by polymerase chain reaction (PCR) screening (Griffin et al., 1984) cells from the TA cloning kit (Invitrogen) were maintained at thermal cycler. Ligations were performed according to Sam-

**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 Hybird 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 Smal digested pUC18 DNA and transformed into *E. coli* MC1022. The constructs were designated pF1730 (2-4 kb insert) and pF1727 (0-8 kb insert) and the strains were designated F17901 and F17939 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 \( \text{ldh} \) of \( L. \) lactis MG1363 with a T in the \( \text{ldh} \) of \( L. \) lactis biovar diacetilactis 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 \( \text{recA} \) genes and \( \text{pepXP} \) genes of \( L. \) lactis and \( L. \) cremoris indicated that a similar comparison of the \( \text{ldh} \) genes might also be of use. In order to perform this comparison the \( \text{ldh} \) 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 \( \text{ldh} \) sequences the gene from \( L. \) lactis IL1403 was also amplified by PCR. Multiple clones were isolated and sequenced. Comparison of the \( \text{ldh} \) gene from \( L. \) lactis IL1403 with that of \( L. \) lactis biovar diacetilactis 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 strains.

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

REFERENCES


Neve, H., Geis, A. & Teuber, M. (1984). Conjugal transfer and...
Comparison of three lactococcal \textit{ldb} genes


Received 20 December 1993; accepted 17 January 1994.