Lactococcus lactis subsp. tructae subsp. nov. isolated from the intestinal mucus of brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss)

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The species Lactococcus lactis currently includes three subspecies; L. lactis subsp. lactis and L. lactis subsp. cremoris, isolated from milk sources, and L. lactis subsp. hordniae, isolated from the leafhopper Hordnia circellata. In this study, three strains, designated L105T, I3 and L101, were isolated from the intestinal mucus of brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss). These strains were closely related to members of the species Lactococcus lactis. Strain L105T showed 99.4% 16S rRNA gene sequence similarity to that of the type strains L. lactis subsp. lactis NCDO 604T and L. lactis subsp. hordniae NCDO 2181T and showed 99.9% similarity to the type strain Lactococcus lactis subsp. cremoris NCDO 607T. Analysis of two housekeeping genes, rpoB and recA, confirmed the close relationship between the novel strains and L. lactis subsp. cremoris with similarities of 99.3 and 99.7%, respectively. The three strains could, however, be differentiated from their closest relatives on the basis of several phenotypic characteristics, as was the case for L. lactis subsp. lactis and L. lactis subsp. hordniae, which were also closely related on the basis of 16S rRNA, rpoB and recA gene sequence similarities. The strains isolated in this study represent a new subspecies, for which the name Lactococcus lactis subsp. tructae subsp. nov. is proposed. The type strain is L105T (=LMG 24662T = DSM 21502T).

The genus Lactococcus currently contains six species isolated from different vegetal, animal and food sources, with L. piscium as the only species hitherto isolated from salmonid fish (Williams et al., 1990). Within the species Lactococcus lactis (Schleifer et al., 1985), three subspecies are currently recognized; L. lactis subsp. lactis and L. lactis subsp. cremoris, isolated from milk sources, and L. lactis subsp. hordniae, isolated from the leafhopper Hordnia circellata (Latorre-Guzmán et al., 1977). Recently, strains of L. lactis subsp. lactis have been also isolated from the intestinal tracts of freshwater fish (Itoi et al., 2008, 2009).

In this study, three novel strains, designated L105T, I3 and L101, were isolated from two different salmonid species, brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss). Phylogenetic analyses based on 16S rRNA, rpoB and recA gene sequences placed these strains within the species Lactococcus lactis but their phenotypic characteristics were different to those of the three subspecies currently recognized in the species. Based on this evidence strain L105T represents the type strain of a new subspecies within Lactococcus lactis, for which the name Lactococcus lactis subsp. tructae subsp. nov. is proposed.

Abbreviations: RAPD, random amplification of polymorphic DNA; REP, repetitive sequence-based.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, rpoB and recA gene sequences of strain L105T are EU770697, GU799692 and GU799681, respectively.

Three supplementary figures and two supplementary tables are available with the online version of this paper.
The three novel strains were isolated from the intestinal mucus of salmonid fish by growth on Man–Rogosa–Sharpe agar (MRS; Pronadisa, Madrid, Spain) at 22 °C for 24 h. Strain L105T was isolated from brown trout and strains L101 and I3 were isolated from rainbow trout. Colonies were white–cream, opaque, round and convex.

The Gram reaction was ascertained by using standard staining methods (Doetsch, 1981). Cells of the three isolates were Gram-reaction-positive, non-sporulating, non-motile cocci, similar to members of other subspecies of Lactococcus lactis.

Amplification and sequencing of the 16S rRNA gene was performed as described by Rivas et al. (2007). The sequences obtained were compared with closely related sequences obtained from GenBank using the BLASTN program (Altschul et al., 1990) and aligned using the Clustal W software (Thompson et al., 1997). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using the neighbour-joining method (Saitou & Nei, 1987), with bootstrap analysis based on 1000 resamplings. The MEGA 4.0 package (Tamura et al., 2007) was used for all analyses.

The 16S rRNA gene sequences of the three strains isolated in this study were identical and thus only that of the type strain L. lactis subsp. lactis NCDO 604T and L. lactis subsp. hordniae NCDO 2181T and 99.9% similarity to the type strain L. lactis subsp. cremoris NCDO 607T. In agreement with these results, strain L105T clustered with L. lactis subsp. cremoris NCDO 607T in the phylogenetic tree (Fig. 1). In this analysis, two strains isolated from fish by Itoi et al. (2009) were included (Supplementary Fig. S1, available in IJSEM Online) showing that they also clustered with L. lactis subsp. lactis and L. lactis subsp. hordniae.

Repetitive sequence-based (REP)-PCR pattern analysis using primer (GTG)5 has previously been shown to be an effective tool for the differentiation of L. lactis subspecies (Rademaker et al., 2007; Svec & Sedláček, 2008). Therefore, we analysed REP-PCR patterns of the novel isolates and the reference strains of each subspecies of L. lactis (Supplementary Fig. S2), according to the methodology described by Marilley et al. (2004). The results showed that strains belonging to the same subspecies displayed the same REP-PCR pattern and that patterns were clearly different among members of different subspecies of L. lactis. Our results confirmed that REP-PCR fingerprinting is a rapid and reliable technique for the differentiation of subspecies within the species L. lactis. In order to analyse the genetic diversity of the three strains isolated in this study, random amplification of polymorphic DNA (RAPD) analysis was performed using the primer M13 (5′-GAGGGTGCC-GGTTCCT-3′), according to Rivas et al. (2006). The results showed that the patterns of strains L105T, L101 and I3 (lanes 7, 8 and 9, respectively; Supplementary Fig. S3) were different. These patterns were also different to those of the type strains of the three recognized subspecies of L. lactis (Supplementary Fig. S3, lanes 1, 3 and 5). The results of the RAPD analysis showed that the three novel strains isolated in this study were genotypically diverse, despite their 16S rRNA gene sequence similarities.

The usefulness of housekeeping genes in bacterial taxonomy and phylogeny has been reported for several bacterial groups (Maiden, 2006) including Gram-positive cocci of the genus Streptococcus, a genus that is phylogenetically close to Lactococcus (Glazunova et al., 2009; Pombert et al., 2009). In this study, the partial sequences of two housekeeping genes, rpoB (~460 nt) and recA (~330 nt), were obtained from type strains of species Lactococcus and from the strains isolated in this study. Gene amplification and sequencing were performed using the primers rpoBlac1F (5′-TACGGKAAAAC-CGTA-3′), rpoBlac1R (5′-TCAARCCAWGCTCCAGG-3′), recAlac1F (5′-GCAGCCTTTATCGATGCTG-3′) and recAIR (5′-GCACGACCACGG-3′), which were designed on the basis of conserved regions of these genes in strains L. lactis subsp. lactis KF147 (accession no. CP001834) and L. lactis subsp. cremoris SK11 (accession no. CP000425). The PCR conditions were as follows: preheating at 95 °C for 9 min, followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 52 °C (recA) or at 47.5 °C (rpoB) for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Results of the phylogenetic analysis of rpoB and recA gene sequences are shown in Figs 2 and 3, respectively. Since this was, to our knowledge, the first study of the rpoB and recA genes in species of genus Lactococcus, there were no previous data relating to similarity levels in these genes among strains of the same species or among different species of the genus.
Nevertheless, results of analyses on members of the genus *Streptococcus*, belonging to the same family as *Lactococcus*, showed that two closely related species, *S. salivarius* and *S. vestibularis*, showed *rpoB* and *recA* gene sequence similarities of 97 and 91 %, respectively, to one another (Drancourt et al., 2004; Glazunova et al., 2009; Pombert et al., 2009). Results obtained between members of different species of the genus *Lactococcus* showed lower similarity values (up to 30 % divergence) in both genes (Figs 2 and 3). These results showed that the *rpoB* and *recA* genes could be highly useful for species differentiation within the genus *Lactococcus*. Moreover, these results showed that the type strains of *L. lactis* subsp. *cremonis* and *L. lactis* subsp. *lactis* had *rpoB* and *recA* gene sequence similarities (97.2 and 91.8 %, respectively) that were at the limit for species differentiation, as was the case for members of the genus *Streptococcus*. The type strain of *L. lactis* subsp. *hordniae* was more closely related to *L. lactis* subsp. *lactis*, with ~99.8 % sequence similarity in both genes (representing one nucleotide difference, in both cases, in the fragment analysed), than to *L. lactis* subsp. *cremonis*, with which it showed ~97.3 and 92 % sequence similarities of ~97.3 and 92 %, respectively. As a group, the three novel strains had an internal similarity level of 100 % in these two genes and, as in the case of the 16S rRNA gene sequence analysis, formed a cluster with *L. lactis* subsp. *cremonis* after the phylogenetic analysis of *rpoB* and *recA* gene sequences (Figs 2 and 3). The sequence similarity values with respect to this subspecies were 99.3 % (*rpoB*) (indicating a difference of three nucleotides in the fragment analysed) and 99.7 % (*recA*) (difference of one nucleotide in the fragment analysed). These data suggested that the three isolates represent a novel subspecies within the species *L. lactis*.

The DNA G + C content of strain L105 was determined by the DSMZ Identification Service. For base composition analysis, DNA was extracted and purified according to Cashion et al. (1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The DNA G+C content of strain L105 was 36.0 mol%, as determined by HPLC according to Mesbah et al. (1989) using a Shimadzu HPLC system. DNA–DNA hybridization analyses were performed according to the method of Ezaki et al. (1989), following the recommendations of Willems et al. (2001). DNA relatedness values between strain L105 and the type strains of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremonis* and *L. lactis* subsp. *hordniae* were 62, 90 and 60 %, respectively. These values were in agreement with the results of the 16S rRNA, *rpoB* and *recA* gene sequence analyses, showing that the three novel strains were more closely related to *L. lactis* subsp. *cremonis* than to *L. lactis* subsp. *lactis* and *L. lactis* subsp. *hordniae*.

Cellular fatty acids were analysed by the DSMZ Identification Service, according to the instructions of the Microbial Identification System (MIDI). The fatty acid profile of strain L105 was similar to that of *L. lactis* according to the data recorded in the MIDI database, which was in agreement with the results of 16S rRNA gene sequence analysis. The major fatty acids detected in strain L105 were C16:0 (37.6 %) and C19:0 cyclo 9c (26.6 %); other fatty acids detected included C14:0 (13.1 %), C18:1ω7c (14.4 %), C18:1ω6c (1.0 %), C20:0ω6c (10.1 %), 11 methyl C18:0ω7c (1.5 %) and summed feature 3 (C16:0ω7c:1ω5cω18:0; 2-OH) (4.3 %). This profile is closest in composition to that of *L. lactis* subsp. *cremonis* than to those of *L. lactis* subsp. *lactis* or *L. lactis* subsp. *hordniae* although there were slight differences in the amounts of various fatty acids (Supplementary Table S1).
Table 1. Characteristics of strains L105T, I3 and L101 and other related taxa of the genus Lactococcus

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*After 24h of incubation in API 20 STREP.
†After 24h of incubation in API 50 CH.
‡Results were positive for strain KCCM 40699T (Cho et al., 2008).

Acid production from carbohydrates was tested using the API 20 STREP and API 50 CH systems according to the manufacturer’s instructions. The results were interpreted following the method of Schleifer et al. (1985). Growth in presence of 4% NaCl was determined using TSA (Difco) as basal medium. For testing antibiotic resistance, the disc diffusion method on sheep blood agar plates (Scharlau Microbiology) was used with the following antibiotics (μg per disc, unless otherwise stated): ampicillin (2), erythromycin (2), ciprofloxacin (5), penicillin (10 IU), polymyxin (300 IU), cloxacillin (1), oxytetracycline (30), gentamicin (10), cefuroxime (30) and neomycin (5) (Becton Dikinson). The type strains of the three subspecies of L. lactis and the reference strains used in REP-PCR and RAPD analyses were included in the phenotypic study. Several differences were noted between the three novel isolates and representatives of the three recognized subspecies of L. lactis (Table 1). Complete results of the antibiotic resistance tests are given in Supplementary Table S2. Biocoding from the API 20 STREP tests gave a result of 7203551 for strains L105T and I3 and 7203511 for strain L101. The two codes matched with L. lactis subsp. lactis at 39% and 87.8%, respectively, in the API AB database. The results of the phenotypic characterization showed that, despite high levels of 16S rRNA, rpoB and recA gene sequence similarity between the three novel strains and reference strains of the other subspecies, the strains were phenotypically diverse and varied in their ability to produce acid from several carbon sources as well as their natural resistance to certain antibiotics. These strains differed from the remaining members of subspecies of L. lactis in several characteristics, as can be seen in Table 1.

Our results confirmed that the subspecies L. lactis subsp. hordniae and L. lactis subsp. lactis, despite being closely related based on the genes analysed in this study, possess phenotypic differences that are sufficient to differentiate between members of L. lactis at the subspecies level, as reported by Schleifer et al. (1985). Similarly, the novel isolates presented many phenotypic differences from their closest phylogenetic relative L. lactis subsp. cremoris that distinguish them as two separate subspecies. The remaining subspecies could be clearly differentiated on the basis of 16S rRNA and housekeeping gene sequence analyses and all subspecies of L. lactis could be differentiated by REP-PCR fingerprinting. Based on the evidence presented in this study, strains L105T, I3 and L101 represent a novel subspecies of Lactococcus lactis, for which the name Lactococcus lactis subsp. tructae subsp. nov. is proposed.
Description of *Lactococcus lactis* subsp. *tructae* subsp. nov.


Characteristics additional to those reported in the original description of the species *Lactococcus lactis* (Schleifer et al., 1985) that allow the differentiation of the novel strains from the remaining subspecies of this species are given below.

Arginine dehydrolase production is positive after 24 h of incubation. Grows in 4% NaCl. Positive for assimilation of gluconate. Acid is produced from maltose, lactose, ribose, mannitol, sucrose and amygdalin but not from *d*-xylose. Acid production from melibiose and raffinose is variable. Sensitive to cefuroxime and resistant to erythromycin and polymyxin B. Resistance to tetracyclin is variable.

The type strain, L105T (=LMG 24662T =DSM 21502T), was isolated from the intestinal mucus of brown trout (*Salmo trutta*). The DNA G+C content of the type strain of the subspecies is 36.0 mol%.

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References


