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 L105^T, 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 L105^T showed 99.4 % 16S rRNA gene sequence similarity to that of the type strains *L. lactis* subsp. *lactis* NCDO 604^T and *L. lactis* subsp. *hordniae* NCDO 2181^T and showed 99.9 % similarity to the type strain *Lactococcus lactis* subsp. *cremoris* NCDO 607^T. 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 L105^T (=LMG 24662^T =DSM 21502^T).

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 L105^T, 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 L105^T 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 L105^T 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 L105^T 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 was analysed and deposited in GenBank. Comparison of complete 16S rRNA gene sequences (1541 bp) with those held in the GenBank database indicated that strain L105^T belonged to the species *Lactococcus lactis*, showing 99.4 % sequence similarity to the type strains *L. lactis* subsp. *lactis* NCDO 604^T and *L. lactis* subsp. *hordniae* NCDO 2181^T and 99.9 % similarity to the type strain *L. lactis* subsp. *cremoris* NCDO 607^T. In agreement with these results, strain L105^T clustered with *L. lactis* subsp. *cremoris* NCDO 607^T 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 & Sedlácek, 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′-GAGGGTGGC-GGTTCT-3′), according to Rivas *et al.* (2006). The results showed that the patterns of strains L105^T, 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 of genus *Lactococcus* and from the strains isolated in this study. Gene amplification and sequencing were performed using the primers rpoBLac1F (5′-TACGGKAAACAC-CGTA-3′), rpoBLac1R (5′-TCAARCCAWGCTCCACGG-3′), recALac1F (5′-GCAGCCTTTATCGATGCTG-3′) and recA1R (5′-GCACGACCACCAGG-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.

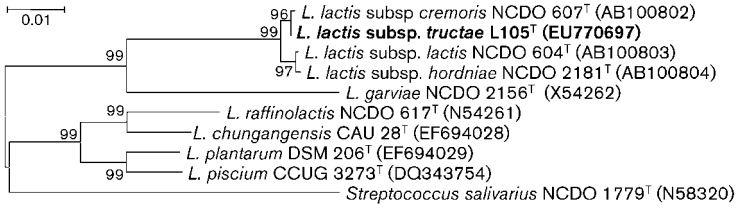

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of strains L105^T, I3 and L101 and other related taxa. Bootstrap values >50%, based on 1000 subsets, are given at branch points. Bar, 0.01 substitutions per nucleotide position.

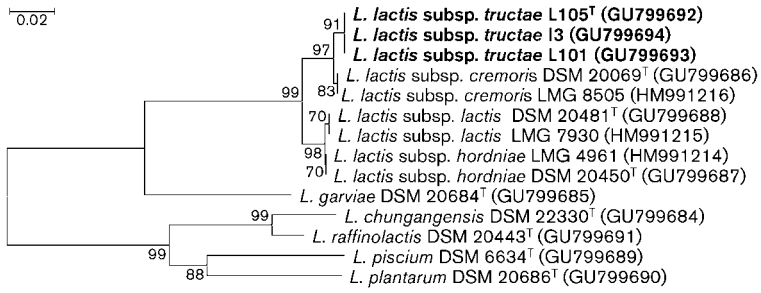

Fig. 2. Neighbour-joining tree based on partial *rpoB* gene sequences of strains L105^T, I3 and L101 and other related taxa. Bootstrap values >50%, based on 1000 subsets, are given at branch points. Bar, 0.02 substitutions per nucleotide position.

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. *cremoris* 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. *cremoris*, with which it showed *rpoB* and *recA* gene 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. *cremoris* 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^T 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^T 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^T and the type strains of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* 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. *cremoris* 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^T 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^T were $C_{16:0}$ (37.6 %) and $C_{19:0}$ cyclo $\omega 8c$ (26.6 %); other fatty acids detected included $C_{14:0}$ (13.1 %), $C_{18:1}\omega 7c$ (14.4 %), $C_{18:0}$ (1.0 %), $C_{20:2}\omega 6,9c$ (1.0 %), 11 methyl $C_{18:1}\omega 7c$ (1.5 %) and summed feature 3 ($C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH) (4.3 %). This profile is closest in composition to that of *L. lactis* subsp. *cremoris* 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).

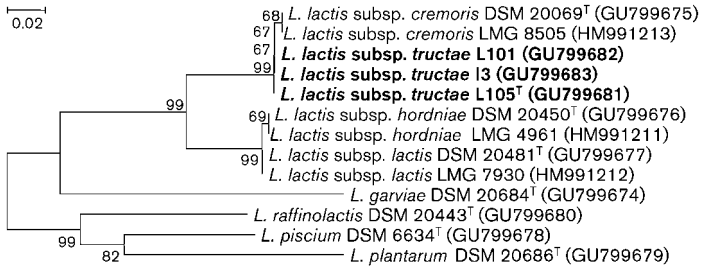

Fig. 3. Neighbour-joining tree based on partial *recA* gene sequences of strains L105^T, I3 and L101 and other related taxa. Bootstrap values >50%, based on 1000 subsets, are given at branch points. Bar, 0.02 substitutions per nucleotide position.

Table 1. Characteristics of strains L105^T, I3 and L101 and other related taxa of the genus *Lactococcus*

Taxa: 1, L105^T; 2, I3; 3, L101; 4, *L. lactis* subsp. *lactis* (DSM 20481^T and LMG 7930); 5, *L. lactis* subsp. *hordniae* (DSM 20450^T and LMG 9462); 6, *L. lactis* subsp. *cremoris* (DSM 20069^T and LMG 8505); 7, *L. chungangensis* CAU 28^T; 8, *L. garvieae* KCTC 3772^T; 9, *L. pisicium* DSM 6634^T (results from Williams *et al.*, 1990; Teuber, 2009); 10, *L. plantarum* DSM 20686^T; 11, *L. raffinolactis* DSM 20443^T. Results for 1–3 and all antibiotic sensitivity tests are from this study. Other results obtained in this study agree with those of Teuber (2009) for taxa 4–6 and 8–11, Schleifer *et al.* (1985) for taxa 4, 5, 6, 8, 10 and 11 and Cho *et al.* (2008) for species 7. +, Positive; −, negative; V, variable; W, weak; ND, no data; S, sensitive; R, resistant; W, weakly resistant.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth in 4 % (w/v) NaCl	+	+	+	+	−	−	−	+	−	+	−
Production of arginine dihydrolase*	+	+	+	+	+	−	−	+	−	−	−
Acid from†:											
Ribose*	+	+	+	+	−	−	ND	+	−	−	V
D-Xylose	−	−	−	+	−	−‡	−	−	+	−	V
Mannitol*	+	+	+	V	−	−	+	−	+	+	−
Lactose*	+	+	+	V	−	+	−	+	+	−	+
Maltose	+	+	+	+	−	−	+	+	+	+	+
Melibiose	+	+	−	−	−	−	−	−	+	−	+
Sucrose	+	+	+	V	+	−	+	−	+	+	+
Raffinose*	+	+	−	−	−	−	−	−	+	−	+
Amygdalin*	+	+	+	W	−	−	+	+	+	+	−
Assimilation of gluconate†	+	+	+	−	−	−	ND	ND	ND	−	−
Resistance/sensitivity to:											
Cefuroxime	S	S	S	R	S	S	S	S	S	S	S
Tetracycline	S	S	R	S	V	W	S	S	S	S	S
Erythromycin	R	R	R	S	S	S	R	R	S	S	S
Polymyxin B	R	R	R	R	S	S	W	R	R	R	W

*After 24h of incubation in API 20 STREP.
†After 24h of incubation in API 50 CH.
‡Results were positive for strain KCCM 40699^T (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 L105^T 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 in 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 L105^T, 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.

Lactococcus lactis subsp. *tructae* (truc′ta.e L. gen. n. *tructae* of a trout fish).

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, L105^{T} (=LMG 24662^{T} =DSM 21502^{T}), 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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