Polyphasic analysis indicates that *Lactobacillus salivarius* subsp. *salivarius* and *Lactobacillus salivarius* subsp. *salicinius* do not merit separate subspecies status

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Lactobacillus salivarius Rogosa *et al.* 1953 was described as a homofermentative lactobacillus with two varieties: *salivarius*, typified inter alia by the ability to ferment rhamnose, and *salicinius*, characterized by the ability to ferment the glucoside salicin. These varieties have become accepted as subspecies divisions. We have examined the relatedness of 32 *L. salivarius* strains by a polyphasic approach. Carbohydrate fermentation profile analysis did not support clear distinction of the two subspecies. *L. salivarius* UCC118 was shown to be facultatively heterofermentative, confirming *in silico* genome analysis. 16S rRNA gene sequences and 16S–23S rRNA intergenic spacer region sequences provided no discrimination between any of the strains or subspecies. Broad subdivisions were distinguishable by pulsed-field gel genomic digest patterns, but they did not allow subspecific or phenotypic distinctions. A phylogeny based upon *groEL* gene sequences was discordant with rhamnose or salicin fermentation data for many taxa, and no reliable phenotypic correlations could be established. In the absence of meaningful taxonomic criteria, we therefore propose that *Lactobacillus salivarius* comprises a single species with no infraspecific taxa. Based on the present study and literature data, an emended description of the species *Lactobacillus salivarius* is provided.

Lactobacillus salivarius was first isolated as part of a survey of human oral lactobacilli (Rogosa *et al.*, 1953) and is commonly found in the oral cavity and gastrointestinal tract of humans (Ahrne *et al.*, 1998; Heilig *et al.*, 2002; Molin *et al.*, 1993; Rogosa *et al.*, 1953) and other animals such as the hamster (Rogosa *et al.*, 1953). In the original study defining the species *L. salivarius*, Rogosa and colleagues appear to have based their classification mainly upon 11 isolates from eight different human subjects, although 106 strains from hamsters were also investigated. *L. salivarius* constituted a clearly separate species from other oral lactobacilli then being characterized, including *Lactobacillus cellobiosus* (Rogosa *et al.*, 1953). Two varieties of *L. salivarius* were recognized in this report, '*Lactobacillus salivarius* var. *salivarius*' and '*Lactobacillus salivarius* var. *salicinius*'. These were distinguished by fermentation profiles. '*L.*

salivarius var. *salivarius*' produced significant acidity in glucose, laevulose, melibiose, trehalose, lactose, mannitol, mannose, raffinose, sucrose, galactose, maltose, rhamnose and sorbitol. It did not ferment arabinose, xylose, cellobiose, melezitose, methyl α -D-glucoside, methyl α -D-mannoside, salicin, glycerol or sorbose (Rogosa *et al.*, 1953). The second variety differed from the 'type variety' in that it fermented salicin but not rhamnose.

No subsequent, more comprehensive investigation of *L. salivarius* has appeared in literature since the original description (Rogosa *et al.*, 1953) and a subsequent 1959 *Lactobacillus* classification paper, which also proposed *L. salivarius* species varieties (Rogosa & Sharpe, 1959). However, Mitsuoka (1969) distinguished five major biotypes among 13 *L. salivarius* strains, which do not correspond readily to the varieties proposed by Rogosa *et al.* (1953). This may be because the strains were from pigs and chickens, as well as humans. The type I biotype most closely resembled '*L. salivarius* var. *salivarius*', but almost half of the salicin-fermenting strains also fermented rhamnose and thus could not be assigned to a subspecies according to the criterion proposed by Rogosa *et al.* (1953). Interestingly, two distinctive biotypes (Va, Vb), which

Abbreviations: ISR, intergenic spacer region; PFGE, pulsed-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains AH4331, DSM 20555^T, NCIMB 702343 and NCIMB 8817 are DQ901732–DQ901735, and the accession numbers of the *groEL* sequences obtained in this study are detailed in Table 1.

ferment ribose, cellobiose and amygdalin, that were previously grouped within the *L. salivarius* species by Mitsuoka (1969), were later reclassified as *Lactobacillus animalis* on the basis of DNA–DNA relatedness (Fujisawa & Mitsuoka, 1996). We recently sequenced the genome of *L. salivarius* subsp. *salivarius* UCC118 (Claesson *et al.*, 2006), a human isolate with probiotic properties (Dunne *et al.*, 1999). This strain ferments rhamnose because it has genes on a megaplasmid (pMP118) that encode rhamnulokinase, L-rhamnose isomerase and rhamnulose-1-phosphate aldolase. The inability of strain UCC118 to ferment salicin is due to the absence of 6-phospho- β -glucosidase, which converts salicin 6-phosphate to glucose 6-phosphate (Claesson *et al.*, 2006).

The two varieties of *L. salivarius* described by Rogosa *et al.* (1953) were included in the Approved Lists of Bacterial Names as subspecies (Skerman *et al.*, 1980; Kandler & Weiss, 1986); the term 'variety' no longer has standing in nomenclature under the *Bacteriological Code* (Rule 5c; Lapage *et al.*, 1992). Culture collection descriptions of the two subspecies also refer to the original isolation. Recent sequence analysis of the 16S–23S rRNA intergenic spacer region (ISR) of the type strains of the two subspecies found them to be 100% identical (Song *et al.*, 2000) and most closely related to *Lactobacillus reuteri*, *Lactobacillus fermentum* and *Lactobacillus plantarum*. The sequences of the ISR flanking the 23S rRNA genes of the two subspecies were also identical. The taxonomic basis for infraspecific subdivision of *L. salivarius* strains was therefore open to question. Sequencing of the genome of *L. salivarius* UCC118 revealed the presence of a complete pentose phosphate pathway (Claesson *et al.*, 2006), including two genes encoding phosphoketolase, a key enzyme involved in heterofermentation (Kandler, 1983). This suggested that this strain is facultatively heterofermentative, which challenges the generally accepted view that *L. salivarius* is an obligately homofermentative lactic acid bacterium. (Badet *et al.*, 2001; Koll-Klais *et al.*, 2005; London, 1976). Preliminary analysis of fermentation end products confirmed that *L. salivarius* UCC118 is indeed heterofermentative (Claesson *et al.*, 2006).

To clarify the infraspecific classification of *L. salivarius*, we employed a polyphasic approach to re-examine the relatedness of strains from both presumptive subspecies. Thirty-two strains from a variety of culture collections were examined (Table 1), which included 15 strains of *L. salivarius* subsp. *salivarius*, 12 strains of *L. salivarius* subsp. *salicinius* and five strains unassigned to a subspecies. For most strains, the rationale for subspecies definition was unclear. Twenty-one strains were of human origin, the remainder, except for a single food isolate, being derived from a variety of mammalian or avian hosts.

The carbohydrate fermenting profile of all strains was determined using API 50 CH strips in conjunction with API 50 CHL medium (bioMérieux). Freshly grown colonies of respective strains were harvested and resuspended in sterile

water to achieve a cell density of 10^{10} c.f.u. ml⁻¹. An aliquot of the cell suspension (200 μ l) was inoculated into 10 ml API 50 CHL medium and mixed gently by inversion; 120 μ l of this suspension was inoculated into API 50 CH strips that were then overlaid with paraffin to maintain anaerobic conditions. Incubation was carried out at 37 °C for 48 h. Fermentation was indicated by a colour change from purple to yellow in the strip cupule. As the inoculum of different strains was adjusted to identical levels, we were able to use the colour change to express the fermentation capabilities semi-quantitatively. The colour changes that we observed were from blue (lack of fermentation) to bottle-green, green, yellow-green, aqua and yellow (complete fermentation). These colour changes were accordingly converted to numerical values in a grey scale (as indicated in the legend to Fig. 1) to facilitate the construction of a dendrogram using BioNumerics software (Applied Maths). This employs the Bray–Curtis method to compare the matrix distance and the UPGMA method to build a similarity tree from the matrix distance (Fig. 1). Seven strains classified as belonging to subspecies *salivarius* did not ferment rhamnose, a supposedly diagnostic criterion, and seven more fermented rhamnose very slowly, with only one rapid rhamnose fermenter. Only one subspecies *salivarius* strain fermented salicin. However, almost half of the supposedly subspecies *salicinius* strains fermented salicin, and five out of 12 strains fermented rhamnose as quickly as the slow subspecies *salivarius* fermenters. None of the carbohydrate fermentation profiles agreed unequivocally with current subspecies strain assignments, nor did any of them suggest a consistent novel subspecies division. Interestingly, ribose fermentation was restricted to three strains, all of which were isolated from humans locally in Cork, Ireland. These strains were grouped in a clearly separated branch from the others, as shown in Fig. 1. Pentose fermentation is usually indicative of heterofermentative ability (Kandler, 1983). We determined the fermentation end products of the sequenced strain UCC118 to validate the correlation between its ribose fermentation and heterofermentative ability. The strain was grown in glucose-free MRS medium supplemented with glucose, fructose or ribose (100 mM each). Following growth at 37 °C for 16 h, the fermentation end products were determined by HPLC. Sampling was performed by taking 1 ml culture supernatant and filtering through a 0.45 μ m sterile syringe filter into a HPLC vial stored on ice. Samples were then analysed on an LKB Bromma 2150 HPLC system equipped with a Shodex RI-71 refractive index detector and a Highchrom heating block. A Rezex 8 μ 8% organic acid column (300 $\times$ 7.8 mm; Phenomenex) was used, with 0.005 M H₂SO₄ as the mobile phase, at a flow rate of 0.6 ml min⁻¹. The temperature of the column was maintained at 65 °C and the injection volume was 20 μ l. While the major end product was still lactate (140 ± 15 mM) when grown on hexoses (glucose or fructose), ethanol (1.8 ± 0.5 mM) was detected when grown on glucose, and ethanol (1.2 ± 0.2 mM) and acetate (4.9 ± 0.4 mM) were detected when grown on fructose. In addition, lactate (125 ± 8 mM), ethanol (2.0 ± 0.3 mM)

Table 1. Strains used in this study

CCUG, Culture Collection of the University of Göteborg; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; LMG, Laboratorium voor Microbiologie, Universiteit Gent; NCIMB, National Collections of Industrial Food and Marine Bacteria. Type strains are indicated by a superscript T.

Strain	Origin	Reference	<i>groEL</i> accession number
<i>Lactobacillus salivarius</i> subsp. <i>salivarius</i>			
UCC118	Human ileal-caecal region	Claesson <i>et al.</i> (2006)	DQ444330
AH4231	Human ileal-caecal region		DQ444346
AH4331	Human ileal-caecal region		DQ444347
AH43310	Human ileal-caecal region		DQ444333
AH43324	Human ileal-caecal region		DQ444334
DSM 20492	Human saliva	Rogosa <i>et al.</i> (1953)	DQ444349
DSM 20555 ^T	Human saliva		DQ444343
NCIMB 8816	Italian human saliva		DQ444359
NCIMB 8817	Turkey faeces		DQ444341
NCIMB 8818	St. Ivel cheese		DQ444332
CCUG 27530B	Human abdomen, abscess		DQ444331
CCUG 38008	Human gall, 73-year-old man		DQ444340
CCUG 43299	Human blood		DQ444356
CCUG 45735	Human blood		DQ444348
CCUG 47825	Human blood, 55-year-old woman		DQ444354
<i>Lactobacillus salivarius</i> subsp. <i>salicinius</i>			
DSM 20554 ^T	Human saliva	Rogosa <i>et al.</i> (1953)	DQ444350
NCIMB 702343	Unknown		DQ444339
CCUG 44481	Bird		DQ444352
CCUG 47171	Human tooth plaque		DQ444337
CCUG 47826	Human blood, 55-year-old woman		DQ444336
JCM 1040	Human intestine	Mitsuoka (1969)	DQ444357
JCM 1042	Human intestine	Mitsuoka (1969)	DQ444360
JCM 1044	Human intestine	Mitsuoka (1969)	DQ444361
JCM 1045	Human intestine	Mitsuoka (1969)	DQ444345
JCM 1046	Swine intestine	Mitsuoka (1969)	DQ444344
JCM 1047	Swine intestine	Mitsuoka (1969)	DQ444351
JCM 1230	Chicken intestine	Mitsuoka (1969)	DQ444335
<i>Lactobacillus salivarius</i> (subspecies unassigned)			
UCC119	Chicken caecum	Woo <i>et al.</i> (2002)	DQ444353
01M14315	Human gall bladder pus		DQ444355
LMG 14476	Cat with myocarditis		DQ444342
LMG 14477	Parakeet with sepsis		DQ444338
L21	Unknown		DQ444358

and acetate (27.0 ± 1.2 mM) were detected when grown on ribose. These data collectively confirm that strain UCC118 is a facultatively heterofermentative *Lactobacillus*, which is consistent with our previous *in silico* genome analysis and preliminary analysis of fermentation products (Claesson *et al.*, 2006). The discovery of *L. salivarius* strains that exhibit heterofermentative metabolism suggests that this species should not be considered as obligately homofermentative.

As the API 50 carbohydrate fermentation profile did not give discriminative separation for subspecies, we employed several molecular techniques in an attempt to identify more discriminatory criteria for the species *L. salivarius*, beginning with pulsed-field gel electrophoresis (PFGE).

Plug preparation of undigested total genomic DNA of lactobacillus strains, macro-restriction digestion and PFGE were carried out as described by Claesson *et al.* (2006). Restriction of genomic DNA with *Sma*I allowed identification of 20 PFGE pattern types for the 32 strains (data not shown). However, assignment of strains to these PFGE types did not concord with subspecies allocation to any extent. To examine ribosomal gene phylogenies, total genomic DNA was isolated by a phenol/chloroform method (Flynn *et al.*, 2002). The primers used for amplifying 16S rRNA genes were 27-F (5'-AGAGTTTGATCMTGGCTCAG) and 1492-R (5'-GGTTACCTTGTTACGACTT) (Gurtler & Stanisich, 1996). All PCR mixtures consisted of 1 ng genomic DNA, 2 mM each dNTP, 2 µM each primer, 0.25 U *Pwo*

Fig. 1. Dendrogram showing the clustering of 32 *L. salivarius* strains based on API 50 carbohydrate fermentation profiles. Numerical value for fermentation capability was defined according to the original colour changes in the API 50 test; values were converted to grey-scale shading for convenience. Names of strains that were previously characterized as *L. salivarius* subsp. *salivarius* are shaded in grey, while those of *L. salivarius* subsp. *salicinius* are shown in bold. Names of strains that are unassigned to a subspecies are underlined. Type strains are indicated by a superscript T.

polymerase and 5 µl *Pwo* polymerase buffer (Roche) in a total volume of 50 µl. PCRs were carried out in a DNA Engine thermal cycler (Bio-Rad). Amplification consisted of initial denaturation (94 °C, 2 min), 30 cycles of denaturation (94 °C, 15 s), annealing (45 °C, 30 s) and elongation (72 °C, 120 s) and a final elongation step (72 °C, 7 min). PCR products were purified using the High Pure PCR purification kit (Roche Diagnostics) and then sequenced by MWG Biotech (Ebersberg, Germany). Apart from the 16S rRNA gene sequence of strain UCC118, which was obtained from the genome sequencing project (Claesson *et al.*, 2006; GenBank accession number CP000233), we sequenced the 16S rRNA genes of strains DSM 20555^T (type strain of *L. salivarius* subsp. *salivarius*), AH4331, NCIMB 8817 and NCIMB 702343. The 16S rRNA gene sequences of some other strains used in this study were already available, including those of strains JCM 1042 (GenBank accession no. AY137584), JCM 1044 (AY137585), JCM 1045 (AY137586), JCM 1046 (AY137587), JCM 1047 (AY137588), JCM 1230 (AY137588) and 01M14315 (AF420311). Of the 12 strains mentioned above, four strains represent the *salivarius*

subspecies and seven strains represent the *salicinius* subspecies, while one strain was unassigned.

The *L. salivarius* UCC118 genome contains seven rRNA operons (Claesson *et al.*, 2006). The sequences of the 16S–23S spacer region of the seven rRNA operons are identical. Although polymorphisms exist between the different 16S rRNA gene copies (three 16S rRNA sequences have one, two and five nucleotide differences compared with the majority consensus), they occur in different positions. We amplified and sequenced the PCR product based on the 16S rRNA genes of *L. salivarius* UCC118 and obtained a sequence chromatogram identical to the consensus, suggesting that sequences of PCR products could be used directly as representative of the consensus sequence of that strain. The 16S rRNA gene sequences of four strains (JCM 1042, JCM 1044, DSM 20555^T and 01M14315) were identical to that of strain UCC118, while the remaining seven strains (JCM 1045, JCM 1046, JCM 1047, JCM 1230, AH4331, NCIMB 8817 and NCIMB 702343) exhibited a single nucleotide difference compared with that of strain UCC118.

Interestingly, we noticed that the sequences of 16S rRNA genes of several *L. salivarius* strains isolated from dog (GenBank accession no. AB186340), horse (AY389802, AY389803, AY389804) and human vagina (AY112743) have more than two nucleotide differences compared with that of strain UCC118. However we could not incorporate these strains into this study, as they were not available to us.

We also amplified and sequenced the 16S–23S ISR of five strains from the *salivarius* subspecies (AH4231, AH4331, UCC119, DSM 20492 and DSM 20555^T), seven strains from the *salicinius* subspecies (DSM 20554^T, JCM 1040, JCM 1042, JCM 1045, JCM 1046, JCM 1047 and JCM 1230) and one unassigned strain (01M14315). The primers used were IGSL (5'-GCTGGATCACCTCCTTTC) and IGSR (5'-CTGGTGCCAAGGCATCCA). All sequences were either identical or differed by a single nucleotide. Song *et al.* (2000) reported that the 16S–23S ISR for the two subspecies type strains of *L. salivarius* (strains DSM 20554^T and DSM 20555^T in Fig. 1) were identical. Our results further show that 16S–23S ISR is not discriminatory for distinguishing strains of *L. salivarius*.

Phylogeny of *groEL* genes has provided superior discriminatory power when applied to many organisms, including *Streptococcus suis* (Brousseau *et al.*, 2001), *Streptococcus mutans* (Hung *et al.*, 2005), pathogenic *Enterococcus* species (Tsai *et al.*, 2005) and *Bifidobacterium* species (Ventura *et al.*, 2004). Phylogeny of the *groEL* gene was exploited to identify novel subspecies in *L. plantarum* (Bringel *et al.*, 2005) and *Lactobacillus delbrueckii* (Dellaglio *et al.*, 2005). The *groEL* and *groES* genes of 32 *L. salivarius* strains including the reference strain UCC118 were amplified using primers LSL_1212groS_F2 (5'-AAACCATTAGGAGATCGCGTT) and LSL_1211groL_R2 (5'-ATCATACCGCCCATACCTG). Consideration of the *groES* gene sequences alone provided poor discrimination (not shown). However, the sequences of *groEL* provided a more reliable basis for constructing a phylogenetic tree.

The *groEL* sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) using default parameters. Phylogenetic trees were built by using either neighbour-joining and maximum-parsimony in MEGA version 3.1 (Kumar *et al.*, 2004) or the method of maximum-likelihood implemented in PhyML (Guindon & Gascuel, 2003) with the general time-reversible plus gamma (GTR+ γ) model (Fig. 2). The phylogenetic trees generated by the different methods exhibited similar topologies, with only minor differences in the positions of some closely related strains. All phylogenetic trees contained three clear divisions, but the maximum divergence seen was modest (24 nucleotides over 1.5 kb of DNA sequence compared). The two type strains, DSM 20554^T (subspecies *salicinius*) and DSM 20555^T (subspecies *salivarius*), were separated into distinct branches, each of which comprised a mixture of strains from the two subspecies. The clade containing the *L. salivarius* subsp. *salivarius* type strain DSM 20555^T was further subdivided into two well-separated clusters. Among the

clades separated, the mean value of the G + C content of the clade containing the *L. salivarius* subsp. *salivarius* type strain DSM 20555^T (36.32 mol%) was the highest, followed by the clade containing strain AH4231 (36.2 mol%) and the clade containing the *L. salivarius* subsp. *salicinius* type strain DSM 20554^T (36.07 mol%). This suggests that the three *groEL* clades experienced unequal nucleotide substitution rates during evolution. However, in contrast to other studies which have provided polyphasic evidence supporting *Lactobacillus* subspecies discrimination (e.g. Bringel *et al.*, 2005), there was no concordance of the *groEL*-based phylogeny with phenotypic traits and the overall level of nucleotide divergence was low. Furthermore, the majority of the nucleotide substitutions were silent, and trees constructed on the basis of amino acid sequences had low bootstrap confidence values (not shown).

In conclusion, members of a diverse panel of *L. salivarius* strains, from multiple culture collections, source animals and isolation dates, could be divided into clades and clusters only on the basis of *groEL* gene phylogeny, a very discriminatory technique. A variety of other methodologies, including rRNA gene comparison and carbohydrate fermentation profiles, did not provide any evidence to support the pragmatic subdivision of this species. We therefore propose that the infraspecific division of *Lactobacillus salivarius* cannot be justified and should be discontinued. There follows an emended description of *Lactobacillus salivarius*, based on previous phenotypic analyses (Mitsuoka, 1969; Rogosa *et al.*, 1953; Rogosa & Sharpe, 1959), the genome sequence (Claesson *et al.*, 2006) and the present study.

Emended description of *Lactobacillus salivarius* Rogosa *et al.* 1953

Cells are Gram-positive, non-motile, non-spore-forming, catalase-negative, nitrate reduction-negative rods with rounded ends, 0.6–0.9 × 1.5–5 µm, occurring singly and in chains of varying length. Sometimes, the cells are not exactly cylindrical and straight, but are instead slightly bent and swollen on one end. Colonies on MRS agar show a milky-white or light-brown colour and are convex, with a smooth edge and smooth surface. Mainly isolated from the mouth and intestinal tract of humans and hamster and the intestinal tract of chicken and swine. All strains grow at 45 °C, but not at 15 °C. Optimal growth temperature is 37 °C. Growth is stimulated in the presence of 5 % CO₂. Glucose, fructose, mannose, mannitol, *N*-acetylglucosamine and sucrose are fermented by all strains. Galactose (30/32 strains tested), sorbitol (28/32), maltose (31/32), lactose (28/32), trehalose (26/32), raffinose (25/32) and melibiose (27/32) are fermented by most strains, including the type strains of the two previously recognized subspecies. Rhamnose is fermented weakly by many strains. A few strains ferment ribose, adonitol, xylitol, D-arabitol, arbutin and salicin and hydrolyse aesculin, but the type strains of the two subspecies do not ferment pentoses or pentitols. Glycerol, erythritol, arabinose, xylose, sorbose, inositol,

Fig. 2. Phylogenetic trees based on the *groEL* gene sequences of 32 *L. salivarius* strains built by using the neighbour-joining (a), maximum-parsimony (b) and maximum-likelihood (c) methods. Numbers at nodes correspond to bootstrap values based on 500 pseudoreplicates. Sequences have been deposited in GenBank; accession numbers are shown in Table 1. Previous subspecies affiliation is indicated as described in Fig. 1. Scale bars show genetic distance.

methyl β -D-xyloside, methyl β -D-mannoside, methyl β -D-gluconoside, amygdalin, cellobiose, melezitose, starch, glycogen, gentiobiose and gluconate are not fermented. The species contains both homofermentative and facultatively heterofermentative strains. Most strains produce L-lactic acid from available hexoses by homofermentation. Heterofermentative end products (lactic acid, acetic acid and ethanol) are produced from hexoses by some strains that can ferment ribose. Some strains have D-lactate dehydrogenase.

The DNA G + C content is 33–36 mol%. Many strains of the species contain megaplasmids of sizes ranging from 100 kb to 380 kb, as well as smaller plasmids. 16S rRNA gene sequences or 16S–23S rRNA ISR sequences do not provide reliable discrimination between strains. Based on *groEL*

phylogeny, the species can be separated into two major clades. Each clade contains one of the type strains of the two previously recognized subspecies, DSM 20554^T (= ATCC 11742^T = JCM 1150^T) (the type strain of *L. salivarius* subsp. *salicinii*) and DSM 20555^T (= ATCC 11741^T = JCM 1231^T) (the type strain of *L. salivarius* and of *L. salivarius* subsp. *salivarius*).

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