Lactobacillus arizonensis is a later heterotypic synonym of *Lactobacillus plantarum*

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The '*Lactobacillus plantarum* group' encompasses the taxa *Lactobacillus plantarum* subsp. *plantarum*, *Lactobacillus plantarum* subsp. *argentoratensis*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus* and *Lactobacillus arizonensis*. In this study, the phylogenetic position of *L. arizonensis* was examined using 16S rRNA gene-specific methodologies (16S rRNA sequencing and ribotyping) and genomic DNA-based investigations [repetitive extragenic palindromic DNA (rep)-PCR and DNA–DNA hybridization]. Our results show that the *L. arizonensis* type strain could not be distinguished from the type strain of *L. plantarum* or from various *L. plantarum* reference strains. Therefore, it is proposed that the species *L. arizonensis* should be reclassified as a heterotypic synonym of *L. plantarum*.

The *Lactobacillus* species *L. plantarum* (subsp. *plantarum* and subsp. *argentoratensis*), *L. paraplantarum*, *L. pentosus* and *L. arizonensis* form a closely related group known as the '*Lactobacillus plantarum* group' (LPG). All of these species are facultatively heterofermentative, produce DL-lactate, contain *meso*-diaminopimelic acid in their cell wall (Curk *et al.*, 1996; Hammes & Hertel, 2003) and are difficult to distinguish from each other by phenotypic characteristics alone. Nevertheless, *L. pentosus* is generally positive for xylose fermentation, whereas *L. arizonensis* and *L. paraplantarum* are unable to ferment this carbohydrate (Kandler & Weiss, 1986; Zanoni *et al.*, 1987; Swezey *et al.*, 2000). Unlike *L. plantarum* and *L. paraplantarum*, *L. pentosus* ferments glycerol but not melezitose (Zanoni *et al.*, 1987; Bringel *et al.*, 1996; Curk *et al.*, 1996).

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Abbreviation: rep-PCR, repetitive extragenic palindromic DNA-PCR.

The GenBank/EMBL/DDBJ accession numbers for the partial nucleotide sequences of the 16S rRNA gene of *L. arizonensis* strains NRRL B-14768^T, NRRL B-14769, NRRL B-14770, NRRL B-14771, NRRL B-14772 and DSM 13273^T are AJ965482, AJ965483, AJ965484, AJ965485, AJ965486 and AJ965487, respectively.

The 16S rRNA gene sequences of the species of the LPG are highly similar (99·7–99·9 %) and, therefore, are also of limited utility in differentiating species (Bringel *et al.*, 1996; Hammes & Hertel, 2003). On the other hand, *L. plantarum*, *L. plantarum* subsp. *argentoratensis*, *L. pentosus* and *L. paraplantarum* can be reliably differentiated from one another by using other molecular techniques. For example, probes targeted against specific genes (*pyrDEF*), were successfully used to differentiate between the closely related species *L. plantarum*, *L. pentosus* and *L. paraplantarum* (Bringel *et al.*, 1996; Quere *et al.*, 1997). DNA–DNA hybridization also clearly distinguishes between *L. plantarum*, *L. pentosus* and *L. paraplantarum* (Dellaglio *et al.*, 1975; Zanoni *et al.*, 1987; Curk *et al.*, 1996).

L. arizonensis is the most recently described member of the LPG (Swezey *et al.*, 2000). It was originally described as a unique LPG species capable of degrading simmondsin at 45 °C and was elevated to species-level status on the basis of this characteristic, as well as 16S rRNA gene sequence divergence and DNA–DNA hybridization data (Swezey *et al.*, 2000). In this study, we show that the type strain of *L. arizonensis* (NRRL B-14768^T = DSM 13273^T) is not distinguishable from the *L. plantarum* type strain (DSM 20174^T) on the basis of ribotyping patterns, repetitive extragenic palindromic DNA (rep)-PCR fingerprinting patterns, 16S

Table 1. LPG strains used in this study

Strain	Source
L. plantarum DSM 20174^T	DSMZ
L. pentosus DSM 20314^T	DSMZ
L. paraplantarum DSM 10667^T	DSMZ
L. arizonensis	
NRRL B-14768^T (=DSM 13273^T)	ARS Culture Collection, (DSMZ)
NRRL B-14769	ARS Culture Collection
NRRL B-14770	ARS Culture Collection
NRRL B-14771	ARS Culture Collection
NRRL B-14772	ARS Culture Collection

rRNA gene sequences and DNA–DNA hybridization data, indicating that the previously described taxon *L. arizonensis* is a later heterotypic synonym of *L. plantarum*.

The bacterial strains used in this study are listed in Table 1. Type strains for *L. plantarum*, *L. pentosus* and *L. paraplantarum* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and several *L. arizonensis* reference strains were obtained from the US Department of Agriculture, Agricultural Research Service (ARS) Culture Collection (formerly known as NRRL). Two preparations of the *L. arizonensis* type strain were also used; these came from the DSMZ and the ARS Culture Collection.

Automated ribotyping was performed using the DuPont Qualicon RiboPrinter system (Bruce, 1996) and with *Eco*RI as the standard restriction enzyme. The band patterns were compared using Bionumerics software (Applied Maths). Clustering was performed by the unweighted pair group method with arithmetic means (UPGMA) (Sneath & Sokal, 1973) based on the Pearson product-moment correlation coefficient, using an optimization coefficient of 1·2 %. The riboprinting patterns obtained from the LPG strains are shown in Fig. 1. In addition to the type strains, additional LPG reference strains were included in order to obtain a broader representation of the LPG species. Strains belonging to *L. pentosus*, *L. paraplantarum* and *L. plantarum* subsp. *argentoratensis* each formed a separate cluster, whereas both of the *L. paraplantarum* strains could be assigned to the same group. Comparison of the latter two fingerprint patterns showed a similarity value of 88·8 %. In contrast, the riboprints of *L. plantarum* and *L. arizonensis* strains could be allocated to three different groups. The first group (1) clustered closely at $r = 89$ % and included the type strain of *L. plantarum* DSM 20174^T, the *L. plantarum* strains DSM 2601, DSM 2648 and DSM 20246, as well as the type strains (NRRL B-14768^T = DSM 13273^T) and strain NRRL B-14769, recently described as *L. arizonensis*. *L. plantarum* strain DSM 20205 formed a separate group (group 2). The remaining strains of *L. arizonensis* (NRRL B-14770, NRRL B-14771, NRRL B-14772), which also clustered closely ($r = 90$ %), formed the third group which also included the strain *L. plantarum* DSM 12028 (group 3; Fig. 1).

Total genomic DNA was isolated from the LPG bacteria listed in Table 1 according to the method of Pitcher *et al.* (1989), as modified by Björkroth & Korkeala (1996), which relies on using a combined lysozyme and mutanolysin treatment. The genomic DNA was used for amplification of the 16S rRNA gene and for rep-PCR fingerprinting. Rep-PCR fingerprinting was conducted using the primer (GTG)5 (5′-GTGGTGGTGGTGGTG-3′) and the methods described by Gevers *et al.* (2001), with slight modifications as described by Kostinek *et al.* (2005). Rep-PCR fingerprints were analysed using the BioNumerics (version 2.5) software package (Applied Maths). Groupings of the rep-PCR fingerprints were performed by using the Pearson product-moment correlation coefficient (r) and the UPGMA clustering algorithm (Sneath & Sokal, 1973).

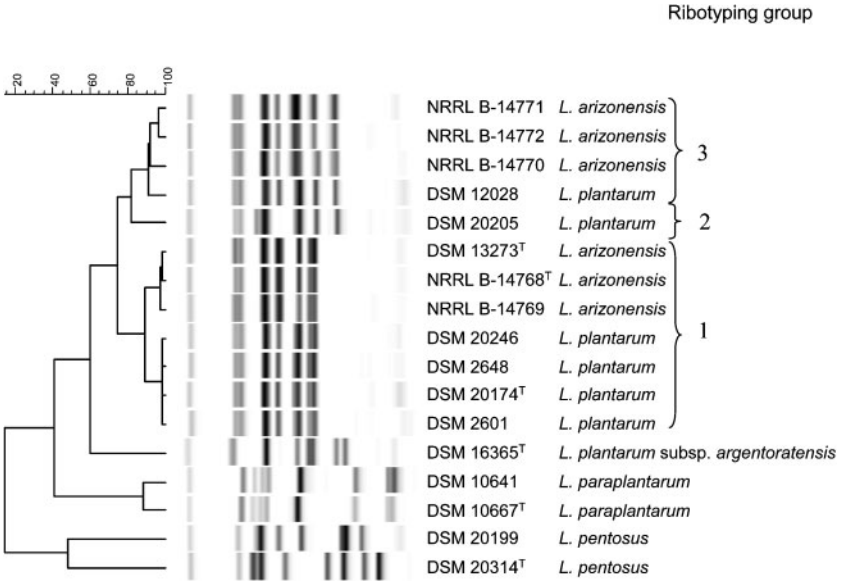

Fig. 1. Cluster analysis of riboprint patterns obtained from strains closely related to *L. plantarum*.

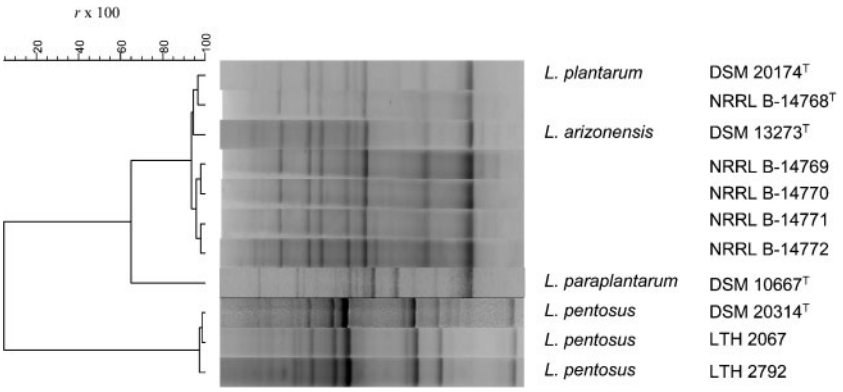

Fig. 2. UPGMA tree obtained from correlation values of rep-PCR fingerprint patterns of *L. arizonensis*, *L. plantarum*, *L. paraplantarum* and *L. pentosus* type and reference strains.

Using rep-PCR, the *L. plantarum* type strain (DSM 20174^T) and *L. arizonensis* type strain (NRRL B-14768^T = DSM 13273^T) grouped closely at $r = 94{\cdot}3\,\%$ (Fig. 2). Furthermore, these grouped closely together with the other *L. arizonensis* strains NRRL B-14769, NRRL B-14770, NRRL B-14771 and NRRL B-14772 at $r = 93{\cdot}6\,\%$. Using rep-PCR, these strains (including the *L. arizonensis* and *L. plantarum* type strains) could be well distinguished from *L. paraplantarum* DSM 10667^T and *L. pentosus* DSM 20314^T, which grouped together with the *L. plantarum*/*L. arizonensis* strains at $r = 64{\cdot}9\,\%$ and $r = 4{\cdot}5\,\%$, respectively (Fig. 2).

The almost-complete 16S rRNA genes of all the LPG strains were amplified and sequenced at GATC Biotech, following the methods of Yousif *et al.* (2005). The 16S rRNA of the *L. arizonensis* type and reference strains was amplified and sequenced independently at the ARS Culture Collection as described previously by Rooney *et al.* (2005). For phylogenetic analysis, the complete 16S rRNA gene sequences were fitted into an alignment of at least 90 % complete primary structure available in public databases using the respective tools of the ARB software package (Ludwig *et al.*, 2004).

16S rRNA gene sequencing showed that the sequence of the *L. arizonensis* strains DSM 13273^T, NRRL B-14768^T, NRRL B-14769, NRRL B-14770, NRRL B-14771 and NRRL B-14772 exhibited high ($>99{\cdot}4\,\%$) similarity to the corresponding 16S rRNA nucleotide sequences of *L. plantarum* DSM 20174^T, *L. pentosus* DSM 20314^T and *L. paraplantarum* DSM 10667^T. In contrast, the level of similarity of these sequences to the 16S rRNA gene sequence deposited in the EMBL nucleotide database for *L. arizonensis* NRRL B-14768^T (GenBank accession number AF093757) was noticeably lower, at 97·2 %. The phylogenetic tree showing the position of strains DSM 13273^T and NRRL B-14768^T compared with their nearest phylogenetic neighbours is shown in Fig. 3. The 16S rRNA gene sequences obtained for strains DSM 13273^T, NRRL B-14768^T, NRRL B-14769, NRRL B-14770, NRRL B-14771 and NRRL B-14772 in this study are almost identical, except for the NRRL B-14772 sequence, which is missing one base (adenine) in the 90–110 region (*Escherichia coli* numbering system), and the sequence of strain NRRL B-14770, which differs in one base in the 180–220 region (*E. coli* numbering system). The phylogenetic analysis shows that the 16S rRNA gene sequence deposited for *L. arizonensis* (AF093757) by Swezey *et al.* (2000) resulted in a noticeably divergent positioning of this species relative to other LPG species (Fig. 3).

LPG taxa are difficult to separate on the basis of 16S rRNA gene sequence similarity. Thus, it is somewhat surprising that the original *L. arizonensis* strain NRRL B-14768^T 16S rRNA gene sequence (GenBank accession number AF093757) displays so many nucleotide differences from the *L. plantarum* and *L. paraplantarum* 16S rRNA gene sequences, as this stands in stark contrast to the otherwise close relatedness in phenotypic and genotypic properties, including 16S rRNA gene sequence relatedness, among other LPG strains and species. Moreover, many of the differences between the original *L. arizonensis* 16S rRNA gene sequence and the other LPG 16S rRNA gene sequences are in conserved regions of the 16S rRNA molecule. This observation suggests that the 16S rRNA gene sequence differences between *L. arizonensis* as originally described and the other LPG species were most likely based on errors. Thus, we believe that the 16S rRNA gene sequence as deposited by Swezey *et al.* (2000) was possibly based on a contaminated culture or sequencing error. According to the results of this study, the 16S rRNA gene sequences of the strains *L. arizonensis* DSM 13273^T and NRRL B-14768^T deposited in both the DMSZ culture collection (GenBank accession number AJ965482) and the ARS Culture Collection clearly represent strains of *L. plantarum*.

For determination of the DNA base composition and DNA–DNA hybridization values, DNA was isolated and purified according to the method of Marmur (1961) as modified by Stackebrandt & Kandler (1979). The DNA base composition (G+C mol%) was determined from the thermal melting temperature (T_m) of DNA using a spectrophotometer (100 Bio UV-Visible; Varian Cary). DNA–DNA relatedness was determined spectrophotometrically from renaturation rates according to De Ley *et al.* (1970). The DNA of representative strains belonging to ribotyping groups 1 and 3 showed DNA–DNA relatedness values with strain NRRL B-14768^T of greater than 70 % (Table 2). Strains NRRL B-14768^T,

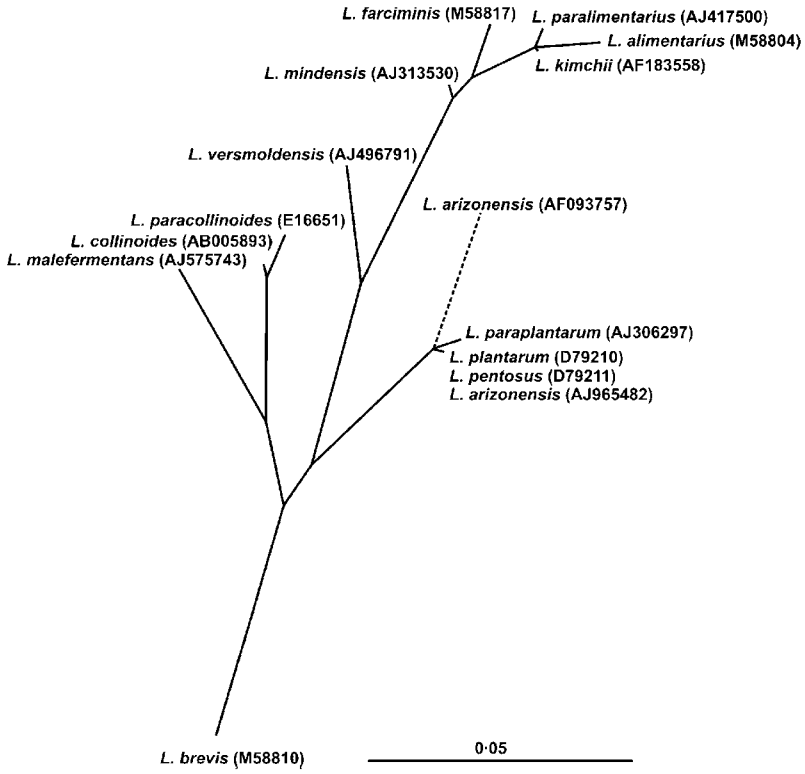

Fig. 3. Phylogenetic tree reflecting the relationship among members of the LPG. The tree is based on a maximum-parsimony analysis of all available at least 90 % complete 16S rRNA gene sequences of the family *Lactobacillaceae*. Alignment positions that share identical residues in at least 50 % of all sequences of the genera *Lactobacillus* and *Pediococcus* were considered. The tree topology was indicated by the positions of the type strain sequences. Bar, 5 % estimated sequence divergence.

NRRL B-14769, DSM 20246, DSM 20174^T and DSM 13273^T all belonged to group 1 in the ribotyping experiments (Fig. 1) and revealed a high DNA–DNA relatedness value of between 73 and 100 % with strain NRRL B-14768^T. Strain NRRL B-14768^T also showed very high DNA–DNA relatedness (96 %) to strain NRRL B-14771, which belonged to group 3 according to the ribotyping results (Fig. 1). DNA–DNA hybridization of strain DSM 13273^T with *L. plantarum* DSM 20174^T (both from ribotyping group 1) resulted in a 100 % relatedness value, while DNA–DNA hybridization of strain NRRL B-14771 (ribotyping group 3) with *L. plantarum* DSM 20174^T (ribotyping group 1) led to a somewhat lower relatedness value of 75 % (result not shown). Furthermore, the DNA–DNA relatedness value detected for strain NRRL B-14771 (ribotyping group 3) and strain DSM 20246 (ribotyping group 1) was 90 % (result not shown).

Table 2. DNA relatedness values to *L. arizonensis* NRRL B-14768^T derived from DNA–DNA hybridization experiments

	Ribotyping group	DNA relatedness value to *L. arizonensis* NRRL B-14768^T (%)*
L. plantarum		
DSM 20246	1	82
DSM 20174^T	1	73
L. arizonensis		
NRRL B-14769	1	94
DSM 13273^T	1	100
NRRL B-14771	3	89
L. pentosus DSM 20314^T	–	53

**L. arizonensis* NRRL B-14768^T is a member of ribotyping group 1, data from this study.

Therefore, strains from both within and between the different ribotyping groups showed DNA–DNA relatedness values of >70 %. This indicated that they are all members of the same species, as a reassociation value of 70 % is considered to be the cut-off value for a species in DNA–DNA hybridization experiments (Wayne *et al.*, 1987). Our DNA–DNA hybridization analyses showed that the *L. arizonensis* and *L. plantarum* type strains, as well as other representative *L. arizonensis* and *L. plantarum* strains, are clearly related at the species level. Previous data by Swezey *et al.* (2000) reported DNA–DNA relatedness values for *L. plantarum* DSM 20174^T and *L. arizonensis* NRRL B-14768^T of 42 %, which is much lower than the value determined in our study. Again, we presume that a mixed culture may possibly have been used in the DNA–DNA hybridization experiments of Swezey *et al.* (2000). However, our results show that the strain submitted to the culture collections is pure and our DNA–DNA hybridization experiments clearly identify the *L. arizonensis* type strain, as deposited in the culture collections, as *L. plantarum*. The DNA–DNA hybridization experiments confirmed the results of our other investigation methods (rep-PCR, ribotyping and 16S rRNA gene sequencing) used to differentiate between the

species. In addition, all our methods could clearly distinguish between the type or reference strains of *L. plantarum*, *L. pentosus* and *L. paraplantarum*. Our investigation clearly shows that the *L. arizonensis* type strain can not be distinguished from the *L. plantarum* type strain and other *L. plantarum* reference strains at the species level. In conclusion, it is proposed that *L. arizonensis* is a later heterotypic synonym of *L. plantarum* and consequently should be renamed.

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