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 paraplantarum 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; Swezy 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).

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 (Bringle 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 (Swezy 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 (Swezy et al., 2000). In this study, we show that the type strain of L. arizonensis (NRRL B-14768T = DSM 13273T) is not distinguishable from the L. plantarum type strain (DSM 20174T) on the basis of ribotyping patterns, repetitive extragenic palindromic DNA (rep)-PCR fingerprinting patterns, 16S


Translated from www.microbiologyresearch.org by
IP: 54.70.40.11
On: 03 Nov 2018 21:30:54

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Published online ahead of print on 29 July 2005 as DOI 10.1099/ij.s.0.63880-0.

Abbreviation: rep-PCR, repetitive extragenic palindromic DNA-PCR.
Table 1. LPG strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>L. plantarum</em> DSM 20174&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td><em>L. pentosus</em> DSM 20314&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td><em>L. paraplantarum</em> DSM 10667&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td><em>L. arizonensis</em> NRRL B-14768&lt;sup&gt;T&lt;/sup&gt; (= DSM 13273&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>ARS Culture Collection, DSMZ</td>
</tr>
<tr>
<td>NRRL B-14769</td>
<td>ARS Culture Collection</td>
</tr>
<tr>
<td>NRRL B-14770</td>
<td>ARS Culture Collection</td>
</tr>
<tr>
<td>NRRL B-14771</td>
<td>ARS Culture Collection</td>
</tr>
<tr>
<td>NRRL B-14772</td>
<td>ARS Culture Collection</td>
</tr>
</tbody>
</table>

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 *EcoRI* 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<sup>T</sup>, the *L. plantarum* strains DSM 2601, DSM 2648 and DSM 20246, as well as the type strains (NRRL B-14768<sup>T</sup> = DSM 13273<sup>T</sup>) 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)<sub>5</sub> (5′-GTGGTGGTGTTG-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).
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*= 94·3 % (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*= 93·6 %. Using rep-PCR, these strains (including the *L. arizonensis* and *L. plantarum* type strains) could be well distinguished from *L. paraplan* L.*tarum* DSM 10667^T^ and *L. pentosus* DSM 20314^T^, which grouped together with the *L. plantarum*/*L. arizonensis* strains at *r*= 64·9 % and *r*= 4·5 %, 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 (>99·4 %) similarity to the corresponding 16S rRNA nucleotide sequences of *L. plantarum* DSM 20174^T^, *L. pentosus* DSM 20314^T^ and *L. paraplan* L.*tarum* 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 (Escherichia 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. paraplan* L.*tarum* 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*<sub>m</sub>) 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^,
NRRL B-14769, DSM 20246, DSM 20174T and DSM 13273T 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-14768T. Strain NRRL B-14768T 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 13273T with *L. plantarum* DSM 20174T (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 20174T 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).

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 20174T and *L. arizonensis* NRRL B-14768T of 42%, which is much lower than the value determined in our study. Again, we assume 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

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

<table>
<thead>
<tr>
<th>Ribotyping group</th>
<th>DNA relatedness value to <em>L. arizonensis</em> NRRL B-14768T (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. plantarum</em></td>
<td></td>
</tr>
<tr>
<td>DSM 20246</td>
<td>1</td>
</tr>
<tr>
<td>DSM 20174T</td>
<td>1</td>
</tr>
<tr>
<td><em>L. arizonensis</em></td>
<td></td>
</tr>
<tr>
<td>NRRL B-14769</td>
<td>1</td>
</tr>
<tr>
<td>DSM 13273T</td>
<td>1</td>
</tr>
<tr>
<td>NRRL B-14771</td>
<td>3</td>
</tr>
<tr>
<td><em>L. pentosus</em> DSM</td>
<td></td>
</tr>
<tr>
<td>20314T</td>
<td>53</td>
</tr>
</tbody>
</table>

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

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**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.
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 cannot 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.

Acknowledgements

We thank H. Kline and J. L. Swezey at the ARS Culture Collection for technical assistance. Financial support from the EU Commission within the framework of the INCO RTD programme is gratefully acknowledged. This study was partly carried out under the project ‘Improving the quality and nutritional status of GARI through the use of starter cultures and fortification with soybean, palm oil and coconut milk’ (ICA4-CT-2002-10058). The work does not necessarily reflect the Commission’s views and in no way anticipates the Commission’s future policy in this area.

References


