Reclassification of *Bacillus axarquiensis* Ruiz-García et al. 2005 and *Bacillus malacitensis* Ruiz-García et al. 2005 as later heterotypic synonyms of *Bacillus mojavensis* Roberts et al. 1994

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The *Bacillus subtilis* group encompasses the taxa *Bacillus subtilis* subsp. *subtilis*, *B. licheniformis*, *B. amylophilaeformiensis*, *B. atrophaeus*, *B. mojavensis*, *B. vallismortis*, *B. subtilis* subsp. *spizizenii*, *B. sonoren*sis, *B. velezensis*, *B. axarquiensis* and *B. malacitensis*. In this study, the taxonomic relatedness between the species *B. axarquiensis*, *B. malacitensis* and *B. mojavensis* was investigated. Sequence analysis of the 16S rRNA gene and the gene for DNA gyrase subunit B (gyrB) confirmed the very high similarities between these three type strains and a reference strain of *B. mojavensis* (> 99 and > 97%, respectively). DNA–DNA hybridization experiments revealed high relatedness values between the type strains of *B. axarquiensis*, *B. malacitensis* and *B. mojavensis* and between these strains and a reference strain of *B. mojavensis* (83–98%). Based on these molecular taxonomic data and the lack of phenotypic distinctive characteristics, *Bacillus axarquiensis* and *Bacillus malacitensis* should be reclassified as later heterotypic synonyms of *Bacillus mojavensis*.

The species *Bacillus subtilis* (subsp. *subtilis* and subsp. *spizizenii*) (Smith et al., 1964; Nakamura et al., 1999), *B. licheniformis* (Skerman et al., 1980), *B. amylophilaeformiensis* (Priest et al., 1987), *B. atrophaeus* (Nakamura, 1989), *B. mojavensis* (Roberts et al., 1994), *B. vallismortis* (Roberts et al., 1996), *B. sonoren*sis (Palmisano et al., 2001), *B. velezensis* (Ruiz-García et al., 2005a), *B. axarquiensis* and *B. malacitensis* (Ruiz-García et al., 2005b), Gram-positive, rod-shaped, endospore-forming, aerobic and fermentative bacteria, are difficult to distinguish from each other by phenotypic characteristics alone, but can be differentiated from one another reliably by using DNA–DNA hybridization.

In the present study, we provide evidence, on the basis of sequence similarities of the 16S rRNA and gyrB genes and DNA–DNA hybridization data, that the type strains of *B. axarquiensis* and *B. malacitensis* are very closely related to the type strain of *B. mojavensis*, suggesting that the three species represent the same species.

The bacterial strains used in this study were cultivated in nutrient agar or broth (Difco) and incubated aerobically at 30°C. Genomic DNA was isolated and purified using the Qiagen Blood & Cell Culture DNA kit. The complete sequence of a 1.5 kb 16S rRNA gene fragment was determined using the MicroSeq Full Gene 16S rDNA bacterial identification kit (Applied Biosystems). Sequencing was performed using an ABI 3730 DNA sequencer (Applied Biosystems and Hitachi). Sequence assembly was performed using the ABI PRISM DNA Sequencing Analysis software (PE Applied Biosystems). Sequence similarity was calculated using the programs of the Wisconsin package version 10.1 (Accelrys Inc.). The sequences obtained in this study and from GenBank were aligned using the CLUSTAL_X program, version 1.8 (Thompson et al., 1997), and phylogenetic analysis was performed using the PHYLIP computer program package.
Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis was made with 1000 replicates except in maximum-likelihood, where only 100 replicates were generated. Tree figures were drawn by TreeView software (Page, 1996).

Sequences of highly conserved housekeeping or other protein-encoding genes provide higher resolution than 16S rRNA gene sequences and may supplant DNA–DNA reassociation or 16S rRNA gene sequence data at the species level for taxonomic analysis (Stackebrandt et al., 2002; Zeigler, 2003). The partial sequence for the gene encoding the subunit B protein of DNA gyrase (gyrB) was included in the phylogenetic analysis for comparative purposes. An approximately 1.2 kb fragment of the gyrB gene was amplified by PCR and sequenced using a set of primers described by Yamamoto & Harayama (1995). Using the method described above, the gyrB gene sequences were also aligned and phylogenetic trees were constructed. Phylogenetic analysis by the neighbour-joining (Fig. 1), maximum-parsimony and maximum-likelihood methods (see Supplementary Figs S1 and S2 available in IJSEM Online) produced similar trees with the exception of minor differences in the tree topology of the basal branches.

The 16S rRNA gene sequences (positions 35–1431; Escherichia coli K-12 numbering) of B. axarquiensis strains LMG 22476T and CIP 108772T, and B. malacitensis strains LMG 22477T and CECT 5687T shared 99.9 % similarity with each other and shared 99.9 % similarity with sequences of B. mojavensis strains BCRC 17124T and BCRC 17531 (Fig. 1). Sequences of the B. axarquiensis and B. malacitensis type strains shared 99.6 and 99.5 % similarity, respectively, with those of B. subtilis subsp. subtilis BCRC 10255T and B. amyloliquefaciens BCRC 11601T. The 16S rRNA gene sequences (positions 35–1431) of B. axarquiensis LMG 22476T and CIP 108772T and B. axarquiensis CR-119T (GenBank accession no. AY603657) shared 99.9, 99.9 and 99.5 % similarity, respectively, with that of B. mojavensis BCRC 17124T. In contrast, the similarity between B. axarquiensis CR-119T and B. mojavensis reported by Ruiz-Garcia et al. (2005b) was noticeably lower, at 97.4 %. The gyrB gene sequences (positions 316–1480; E. coli K-12 numbering) for B. axarquiensis LMG 22476T and CIP 108772T and B. malacitensis LMG 22477T and CECT 5687T showed high similarities (>97 %) with the investigated B. mojavensis strains and significantly lower similarities (ranging from 80 to 90 %) with their nearest neighbour (Fig. 1b). The similarities of the 16S rRNA and gyrB gene sequences indicate that B. axarquiensis, B. malacitensis and B. mojavensis cannot be differentiated and may represent the same species.

The G+C content was determined using reversed-phase HPLC according to Tamaoka & Komaga (1984) with slight modifications. The nucleoside mixture was separated using a Cosmosil 5C$_{18}$ column (Waters, 4.0 × 150 mm; Nacalai Tesque) in a mobile phase composed of 0.2 M (NH$_4$)$_2$HPO$_4$/acetonitrile (20:1, v/v) at a flow rate of 1 ml min$^{-1}$ at room temperature. DNA relatedness values between B. axarquiensis LMG 22476T and CIP 108772T, B. malacitensis LMG 22477T and CECT 5687T, B. mojavensis strains BCRC 17124T and BCRC 17531, B. subtilis subsp. subtilis BCRC 10255T and B. amyloliquefaciens BCRC 11601T were determined using the fluorometric hybridization method in microdilution wells as described previously (Ezaki et al., 1989; Goris et al., 1998; Chern et al., 2004). The hybridization temperature was 42 °C. DNA G+C contents of B. axarquiensis (LMG 22476T and CIP 108772T), B. malacitensis (LMG 22477T and CECT 5687T) and B. mojavensis strains BCRC 17124T and BCRC 17531 were respectively 43, 44 and 44 mol% These values were close to the value of 43 mol% determined for the type strain of B. mojavensis by Roberts et al. (1994). The relatedness values of genomic DNA of the strains tested are shown in the Table 1. B. axarquiensis LMG 22476T and CIP 108772T, B. malacitensis LMG 22477T and CECT 5687T and B. mojavensis BCRC 17124T and BCRC 17531 showed high DNA–DNA relatedness values, in the range 83–98 %, indicating clearly that these taxa belong to the same genospecies. These strains showed less than 40 % relatedness with B. subtilis subsp. subtilis BCRC 10255T, B. vallismortis BCRC 17183T and B. amyloliquefaciens BCRC 11601T. The results contradict those obtained by Ruiz-Garcia et al. (2005b), who reported a low hybridization value of less than 47 % between the type strains of these taxa.

Cellular fatty acid composition was determined using the Sherlock Microbial Identification System (MIDI Inc.). Extracts of methylated fatty acids were prepared according to the protocol provided by the manufacturer and analysed with an Agilent 6890N gas chromatograph equipped with a flame-ionization detector and 7683 Automatic Liquid Sampler (Agilent). Identification of peaks was made by comparing the results with the built-in TSBA 50 database (MIDI Inc.). The fatty acid compositions of phylogenetically related species are listed in Supplementary Table S1 (available in IJSEM Online). In strains of B. axarquiensis, B. malacitensis and B. mojavensis, iso- and anteiso-fatty acids with 15 to 17 carbons predominated. Their fatty acids profiles showed high similarity, and could not be used to differentiate the strains from one another or from B. subtilis. The results differed from those obtained by Ruiz-Garcia et al. (2005b), who reported a significant quantity (11.5 %) of 16:0 fatty acid in B. axarquiensis CR-119T and no unsaturated fatty acids in B. axarquiensis CR-119T or B. malacitensis CR-95T.

Additional biochemical tests were performed using the API CH50 and 20E kits (bioMérieux) according to the manufacturer’s instructions. Supplementary Table S2 shows differential biochemical features between the tested Bacillus strains. Very few of these characteristics could be used to differentiate B. axarquiensis and B. malacitensis from one another or from the other species. Only a negative oxidase
Fig. 1. Neighbour-joining trees based on 16S rRNA (a) and gyrB (b) gene sequences of Bacillus strains. Genetic distances were computed by Kimura’s two-parameter model. Escherichia coli K-12 was used as an outgroup. Only bootstrap percentages above 50% are shown. Bars, 0.01 (a) and 0.1 (b) substitutions per nucleotide position.
**Table 1. DNA–DNA relatedness values between Bacillus strains**

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<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>Relatedness (%) to labelled DNA from:</th>
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<tr>
<td></td>
<td>B. axarquiensis LMG 22476&lt;sup&gt;T&lt;/sup&gt;</td>
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<tr>
<td>B. axarquiensis LMG 22476&lt;sup&gt;T&lt;/sup&gt; (= BCRC 17501&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>100</td>
</tr>
<tr>
<td>B. axarquiensis CIP 108772&lt;sup&gt;T&lt;/sup&gt; (= BCRC 17653&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>99</td>
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<tr>
<td>B. malacitensis LMG 22477&lt;sup&gt;T&lt;/sup&gt; (= BCRC 17502&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>87</td>
</tr>
<tr>
<td>B. malacitensis CECT 5687&lt;sup&gt;T&lt;/sup&gt; (= BCRC 17654&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>84</td>
</tr>
<tr>
<td>B. mojavensis BCRC 17124&lt;sup&gt;T&lt;/sup&gt; (= DSM 9205&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>97</td>
</tr>
<tr>
<td>B. mojavensis BCRC 17531 (= DSM 9206)</td>
<td>84</td>
</tr>
<tr>
<td>B. subtilis subsp. subtilis BCRC 10255&lt;sup&gt;T&lt;/sup&gt; (= ATCC 6051&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>31</td>
</tr>
<tr>
<td>B. vallismortis BCRC 17183&lt;sup&gt;T&lt;/sup&gt; (= DSM 11031&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>35</td>
</tr>
<tr>
<td>B. amyloliquifaciens BCRC 11601&lt;sup&gt;T&lt;/sup&gt; (= ATCC 23350&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>15</td>
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**test and positive reactions for acid production from gentiobiose and inulin differentiated** B. *axarquiensis* from B. *malacitensis* and B. *mojavensis*. Additionally, B. *axarquiensis* LMG 22476<sup>T</sup> and CIP 108772<sup>T</sup> are both negative for arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities (not shown in Supplementary Table S2), which differed from the results obtained by Ruiz-García et al. (2005b).

Although RAPD analysis with primers OPA3 and OPL12 could be used to distinguish B. *axarquiensis* and B. *malacitensis* from one another and from B. *mojavensis* (Ruiz-García et al., 2005b), genetic heterogeneity in the same species has also been reported by some authors (Tynkkynen et al., 1999; Matarante et al., 2004; Naser et al., 2006).

Previous data reported by Ruiz-García et al. (2005b) gave DNA–DNA relatedness values for B. *axarquiensis* and B. *malacitensis* strains with strains of other closely related species that were lower than 46.9%, which is much lower than the values (83–99%) determined in our study. Our results show that the strains submitted to the culture collections are pure, and our DNA–DNA hybridization experiments clearly identify the B. *axarquiensis* and B. *malacitensis* type strains, as deposited in the culture collections, as B. *mojavensis*. The DNA–DNA hybridization experiments confirmed the results of our other investigation methods (16S rRNA and gyrB gene sequencing) used to differentiate between the species. In addition, these methods could clearly distinguish between type or reference strains of other closely related species. Our investigation shows clearly that the B. *axarquiensis* and B. *malacitensis* type strains can not be distinguished from the B. *mojavensis* type strain or another B. *mojavensis* reference strain at the species level. The data from the present study show that no significant phenotypic or genotypic differences exist to justify the separation of the type strains of B. *axarquiensis* and B. *malacitensis* and that of B. *mojavensis*. Consequently, it is proposed that *Bacillus axarquiensis* Ruiz-García et al. 2005 and *Bacillus malacitensis* Ruiz-García et al. 2005 should be considered as later heterotypic synonyms of *Bacillus mojavensis* Roberts et al. 1994.

**Emended description of Bacillus mojavensis Roberts et al. 1994**

The description is the same as that given by Roberts et al. (1994) except for the following traits. Oxidase, citrate utilization and nitrite reduction are variable. Does not hydrolyse L-rhamnose or D-galactose. Acid production from D-xylose, inositol, inulin, D-raf-finoose, gentiobiose, amygdalin and methyl a-D-glucoside is strain-dependent. Major fatty acids are 15:0 anteiso, 15:0 iso, 17:0 iso and 17:0 anteiso. The DNA G+C content ranges from 43 to 44 mol%. Known habitats are desert soils and brackish sediments. Additional chemotaxonomic data and results of biochemical tests are given in Supplementary Tables S1 and S2.

The type strain is strain RO-H-1<sup>T</sup> (= NRRL B-14698<sup>T</sup> = DSM 9205<sup>T</sup> = LMG 17797<sup>T</sup> = CIP 104095<sup>T</sup> = ATCC 51516<sup>T</sup> = BCRC 17124<sup>T</sup> = NBRC 15718<sup>T</sup>).

**Acknowledgements**

We thank T. Y. Liu, C. C. Liao and G. F. Yuan (Food Industry Research and Development Institute, Taiwan) for their encouragement. This research was supported by the Taiwanese Ministry of Economic Affairs (project no. 95-EC-17-A-17-R7-0525).

**References**


