Species identification of genus *Bifidobacterium* based on partial HSP60 gene sequences and proposal of *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov.

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Sequence homology of partial 60 kDa heat-shock protein (HSP60) genes was analysed for 50 *Bifidobacterium* strains that represent 12 *Bifidobacterium* species and subspecies with validly published names. Sequence similarities were 96.5–100 % within the same species, 95.5–97 % at the subspecies level and 80–96 % (mean, 88 %) at the interspecies level among the 10 *Bifidobacterium* species. Hence, the HSP60 gene was a more accurate tool for species identification within the genus *Bifidobacterium* than 16S rDNA. Two new *Bifidobacterium* strains isolated from piglet faeces were shown to be closely related to the thermophilic bifidobacterial group, based on 16S rDNA sequence analysis: strain P3-14T (= AS 1.3009T = LMG 21689T) exhibited 97–99 % similarity to *Bifidobacterium boum* JCM 1211T, 97–2 % similarity to *Bifidobacterium thermacidophilum* AS 1.2282T and 97 % similarity to *Bifidobacterium thermophilum* JCM 1207T. However, higher levels of DNA–DNA relatedness (83 %) and HSP60 gene sequence similarity (97 %) were determined between *B. thermacidophilum* AS 1.2282T and strain P3-14T, indicating a closer relationship between them. The new strains differed from *B. thermacidophilum* AS 1.2282T in some phenotypic characteristics, such as growth at a lower temperature (46.5 °C), as well as different sugar-fermentation patterns. Hence, a novel *Bifidobacterium* subspecies, *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov., is designated.

Bifidobacteria are obligately anaerobic bacteria and a member of the commensal intestinal microflora of humans and animals. Currently, the genus *Bifidobacterium* comprises over 30 species with validly published names, one of which also contains subspecies (Scardovi, 1986; Lauer, 1990; Biavati & Mattarelli, 1991; Biavati et al., 1991; Crociani et al., 1996; Meile et al., 1997; Dong et al., 2000; Hoyles et al., 2002; Jian & Dong, 2002). Conventional identification and classification of *Bifidobacterium* species have relied heavily on carbohydrate-fermentation patterns, which have been demonstrated to be strain-specific, rather than species-specific (Roy & Ward, 1990).

16S rDNA homology analysis has been demonstrated to be a powerful and accurate method to determine phylogenetic relationships among bacteria, including generic relationships (Olsen et al., 1994; Stackebrandt & Goebel, 1994). Sequence similarity levels among *Bifidobacterium* species ranged from 93 to 99 % and were even greater than 99 % within five groups (Miyake et al., 1998); this is too high to distinguish between *Bifidobacterium* species.

DNA–DNA hybridization has been acknowledged to be a superior method to elucidate relationships between closely related taxa, such as species. Bifidobacterial members within a species generally exhibited DNA–DNA reassociation values of >70 % (Lauer & Kandler, 1983). However, this is a laborious and inconvenient method to identify an unknown isolate.

Transaldolase gene sequence-based PCR-denaturing gradient gel electrophoresis (PCR-DGGE) patterns have also been used to differentiate bifidobacterial species isolated from humans (Requena et al., 2002); however, this method failed to discriminate *Bifidobacterium catenulatum* from *Bifidobacterium angulatum*.

**Abbreviation:** HSP, heat-shock protein.

The GenBank/EMBL/DDBJ accession numbers for the partial HSP60 gene and 16S rDNA sequences of *Bifidobacterium* species and the complete 16S rDNA sequence of *Bifidobacterium thermacidophilum* subsp. *porcinum* reported in this study are AY166508–AY166575 and AY148470, respectively.

A list of *Bifidobacterium* strains used in this study and GenBank accession numbers for their HSP60 and 16S rRNA gene sequences is available as supplementary material in IJSEM Online.
The highly conserved 60 kDa heat-shock protein (HSP60) has been considered to be another useful phylogenetic marker and sequence comparisons of its gene have been used for species identification and phylogenetic analysis of the genera *Staphylococcus* (Kwok *et al*., 1999) and *Bartonella* (Marston *et al*., 1999). In our previous work (Jian *et al*., 2001), it was indicated that the HSP60 gene may also be a useful tool for phylogenetic study of the genus *Bifidobacterium*. The clustering pattern was not only similar to that of 16S rDNA, but there was also a better correlation with DNA G+C content. Furthermore, HSP60 gene sequence similarities (~84–96%) between *Bifidobacterium* species were much lower than those of 16S rDNA.

To evaluate the feasibility of using HSP60 genes to identify *Bifidobacterium* species, 32 *Bifidobacterium* strains that represented eight species and subspecies were isolated from faeces of several kinds of animal (see supplementary material in IJSEM Online) by using Hungate’s anaerobic roll-tube technique with TPYG (trypticase/peptone/yeast extract/glucose) medium (Scardovi, 1986). Gram-stain, anaerobic growth and catalase and fructose-6-phosphate phosphoketolase (F6PPK) activities were detected as described by Scardovi (1986). End-products of glucose fermentation were detected by GC using a model GC-14B chromatograph (Shimadzu). Biochemical characteristics were determined by using an API 50CH kit (bioMérieux). Temperature profiles were determined by using a water bath with a temperature gradient.

To confirm the identification of the 32 new isolates phylogenetically, DNA was prepared and purified as described by Marmur (1961) and 16S rDNA was amplified by PCR and sequenced as described previously (Jian *et al*., 2001). Partial HSP60 gene sequences were also translated into protein sequences by using the PRIMER PREMIER program, version 5.00 (Biosoft International). Methods for sequence similarity calculation and phylogenetic tree construction were described previously (Jian *et al*., 2001).

DNA G+C content was determined by thermal denaturation (Marmur & Doty, 1962). DNA–DNA relatedness between the new isolates and reference strains was determined by using the reassociation rate method (Dong *et al*., 2000).

In this work, partial HSP60 genes were sequenced for 37 *Bifidobacterium* strains that represented 10 *Bifidobacterium* species, including two subspecies, with validly published names. HSP60 gene sequence homology was analysed totally for 50 bifidobacterial strains, including 12 type strains, and another *Bifidobacterium infantis* strain that was studied previously; a phylogenetic tree (Fig. 1) was constructed. The result showed that partial HSP60 gene sequence similarity was 96–5–100% (mean, 98%) among strains within a species, 95–5–97% between *Bifidobacterium pseudolongum* subsp. *pseudolongum* DSM 10140T and nine strains of *B. pseudolongum* subsp. *globosum* and 80–96% (mean, 88%) among the 10 *Bifidobacterium* species in this study. The result was in agreement with the previous conclusion (Jian *et al*., 2001).

Noticeably, HSP60 gene sequence similarities were determined to be 98–100% among eight isolates of *B. infantis*, five isolates of *B. longum* and two isolates of *Bifidobacterium suis*, a similarity level equivalent to those within other *Bifidobacterium* species. Meanwhile, at 99% HSP60 gene sequence similarity, these strains were clustered into three groups. The results supported the proposal to unify *B. infantis*, *B. longum* and *B. suis* into one species as three biotypes (Sakata *et al*., 2002), based on high 16S rDNA sequence similarities (≥99%) among them.

An extremely high 16S rDNA sequence similarity (98.8%) was also determined between *Bifidobacterium lactis* and *Bifidobacterium animalis*; Cai *et al.* (2000) proposed that *B. lactis* is a junior subjective synonym of *B. animalis*, based on this high 16S rDNA sequence similarity and high DNA–DNA hybridization (85.5–92.3%) between the type strains of the two species. In the current study, high partial HSP60 sequence similarity was determined among five *B. animalis* and two *B. lactis* strains: 98–9–100% within group 1 (including *B. lactis* DSM 10140T, *B. lactis* JB-1 and *B. animalis* B83) and group 2 (including *B. animalis* JCM 1190T, *B. animalis* D1, *B. animalis* III-1 and *B. animalis* III-6); however, similarity levels ranged from 97.0 to 97.8% between the two groups. Hence, *B. lactis* DSM 10140T and other *B. lactis* strains should be regarded as another subspecies of *B. animalis*, although they were genetically and phenotypically very similar to some *B. animalis* strains.

Partial HSP60 gene sequence similarity between five *Bifidobacterium pseudocatenulatum* and three *B. catenulatum* isolates (91–93%) supported their interspecies relationship, although they had extremely high 16S rDNA gene sequence similarity (99.5%).

Combining the results of this work and the previous study (Jian *et al*., 2001), a conclusion could be drawn that homology of the partial HSP60 gene of the bifidobacteria was 80–96%, 96–5–100% and 95–5–97% at the interspecies, intraspecies and inter-subspecies levels, respectively. Deduced HSP60 protein sequence similarities were 87–100%, 98–5–100% and 98–100% at the interspecies, intraspecies and inter-subspecies levels, respectively. Compared to 16S rDNA, the HSP60 gene has sufficient power for identification of *Bifidobacterium* species.
Deduced HSP60 protein sequences showed 100% similarity among the thermophilic group that comprises *B. boum*, *B. thermacidophilum* and *B. thermophilum*, although interspecies DNA homologies were 92.9–95.5%. Three signature amino acid residues at positions 156, 158 and 179 (Lys, Arg and Ser) differentiated this group.
from all other bifidobacteria (Asn, Lys and Thr) tested. Shared primary structures of HSP60 might relate to their thermophilic characteristics.

Based on HSP60 gene sequence similarities, two new isolates from piglet faeces, P3-14T and P3-11, were identified as a novel subspecies of *B. thermacidophilum*. Similarities between strain P3-14T and others were determined as follows: 97 % with *B. thermacidophilum* AS 1.2282T, 95-5 % with *B. thermophilum* JCM 1207T and 94-4 % with *B. boum* JCM 1211T.

Homology analysis of the complete 16S rDNA sequence (1523 bp) of P3-14T also indicated its affiliation to the thermophilic bifidobacteria: *B. boum* (97-7 % similarity), *B. thermacidophilum* (97-2 % similarity) and *B. thermophilum* (97-0 % similarity). Furthermore, DNA–DNA relatedness was determined to be 83-0, 58-6 and 25-5 % between isolate P3-14T and *B. thermacidophilum* AS 1.2282T, *B. thermophilum* JCM 1207T and *B. boum* JCM 1211T, respectively, indicating that the new isolate belonged to *B. thermacidophilum*.

However, compared to *B. thermacidophilum*, strain P3-14T grew at a relatively low temperature, as well as the fact that it fermented trehalose but not D-mannose, inulin or L-arabinose. Table 1 shows differential phenotypic characteristics of strain P3-14T, *B. thermophilum* JCM 1207T, *B. thermacidophilum* AS 1.2282T and *B. boum* JCM 1211T. Considering the relatively low 16S rDNA and HSP60 gene sequence similarities, different G + C contents and fermentation patterns among them, as well as the lower growth temperature of P3-14T, a novel subspecies, *Bifidobacterium thermacidophilum* subspp. *porcinum* subspp. nov., is proposed. Accordingly, *B. thermacidophilum* is assigned as *B. thermacidophilum* subspp. *thermacidophilum*.

### Table 1. Differential phenotypic characteristics of *B. thermacidophilum* subspp. *porcinum* subspp. nov., *B. thermacidophilum*, *B. thermophilum* and *B. boum*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
<td>Fermentation of:</td>
<td></td>
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<tr>
<td>L-Arabinose</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
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<td>D-Mannose</td>
<td>+</td>
<td>−</td>
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<td>+</td>
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<td>w</td>
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<td>−</td>
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<td>w</td>
<td>D</td>
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<tr>
<td>Trehalose</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>D</td>
</tr>
<tr>
<td>Maximum growth temp (°C)</td>
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<td>DNA G+C content (mol%)</td>
<td>61-5</td>
<td>57-7</td>
<td>60</td>
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</tr>
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</table>

**Emended description of Bifidobacterium thermacidophilum (Dong et al. 2000)**

*Bifidobacterium thermacidophilum* (therm.a.ci.do’phi.lum. Gr. n. therme heat; N.L. n. acidum acid; N.L. adj. philum from Gr. adj. philos loving; N.L. adj. thermacidophilum heat- and acid-loving).

Gram-positive, non-motile, non-spore-forming, irregular rods, 0-5 × 3-8 μm in size after 12–24 h growth in TPYG medium at 37 °C; strictly anaerobic, no gas is formed from glucose. Optimum temperature for growth is 37–41 °C; temperature range for growth is 30–49-5 °C. Optimum initial pH is 7-0–7-2, growth is quite good at pH 4-5 but is delayed at pH 4-0. D-Fructose, galactose, D-glucose, maltose, melibiose, sucrose, D-raffinose, amidon, glycogen and D-turanose are fermented. Mannitol, rhamnose and sorbose are not fermented. Fermentation of L-arabinose, glucanate, inulin, lactose, D-mannose, methyl z-D-glucoside, ribose, salicin, trehalose and xylose is variable. Litmus milk is not acidified or coagulated by most strains. Fermentation products from glucose are acetic and lactic acids, at a molar ratio of 2:46–4:9 : 1. DNA G + C content is 54-8–61-9 mol%.

**Description of Bifidobacterium thermacidophilum subsp. thermacidophilum subsp. nov.**

All strains meet the species description. Maximum temperature for growth is 49-5 °C. L-Arabinose, inulin and D-mannose are fermented. Trehalose is not fermented. Fermentation products from glucose are acetic and lactic acids at a molar ratio of 2:46–2:72 : 1. DNA G + C content is 56-85 ± 2-05 mol%.

The type strain, 36T (= AS 1.2282T = LMG 21395T), was isolated from wastewater.

**Description of Bifidobacterium thermacidophilum subsp. porcinum subsp. nov.**

*Bifidobacterium thermacidophilum* subsp. *porcinum* (por. ci’num. L. neut. adj. porcinum of a hog).

All strains meet the species description. Maximum temperature for growth is 46-5 °C. Trehalose is fermented. L-Arabinose, inulin and D-mannose are not fermented. Fermentation products from glucose are acetic and lactic acids at a molar ratio of 4:9 : 1. DNA G + C content is 61-2 ± 0-7 mol%.

The type strain, P3-14T (= AS 1.3009T = LMG 21689T), was isolated from the faeces of a piglet.

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