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 P3-14^T (= AS 1.3009^T = LMG 21689^T) exhibited 97.9 % similarity to *Bifidobacterium boum* JCM 1211^T, 97.2 % similarity to *Bifidobacterium thermacidophilum* AS 1.2282^T and 97 % similarity to *Bifidobacterium thermophilum* JCM 1207^T. However, higher levels of DNA–DNA relatedness (83 %) and HSP60 gene sequence similarity (97 %) were determined between *B. thermacidophilum* AS 1.2282^T and strain P3-14^T, indicating a closer relationship between them. The new strains differed from *B. thermacidophilum* AS 1.2282^T 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).

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.

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*.

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 by using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kits (PerkinElmer). The most similar 16S rDNA sequences in GenBank were found by searching with the BLAST program (version 2.2.1). Species identification and GenBank accession numbers of 16S rDNA are available as supplementary material in IJSEM Online.

The partial HSP60 gene (600 bp) was amplified, cloned 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* JCM 1205^T 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 10140^T, *B. lactis* JB-1 and *B. animalis* B83) and group 2 (including *B. animalis* JCM 1190^T, *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 10140^T 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.

Fig. 1. Phylogenetic tree based on fragments (538 bp) of HSP60 gene DNA sequences. The tree was rooted with *Bacillus subtilis* W168 and constructed by using the Fitch method with bootstrap values calculated from 1000 trees. Numbers at each branch-point represent bootstrap support (%); GenBank accession numbers of HSP60 gene sequences are given in parentheses. Bar, 5% sequence divergence.

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, P3-14^T and P3-11, were identified as a novel subspecies of *B. thermacidophilum*. Similarities between strain P3-14^T and others were determined as follows: 97 % with *B. thermacidophilum* AS 1.2282^T, 95.5 % with *B. thermophilum* JCM 1207^T and 94.4 % with *B. boum* JCM 1211^T.

Homology analysis of the complete 16S rDNA sequence (1523 bp) of P3-14^T 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 P3-14^T and *B. thermacidophilum* AS 1.2282^T, *B. thermophilum* JCM 1207^T and *B. boum* JCM 1211^T, respectively, indicating that the new isolate belonged to *B. thermacidophilum*.

However, compared to *B. thermacidophilum*, strain P3-14^T 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 P3-14^T, *B. thermophilum* JCM 1207^T, *B. thermacidophilum* AS 1.2282^T and *B. boum* JCM 1211^T. 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 P3-14^T, a novel subspecies, *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov., is proposed. Accordingly, *B. thermacidophilum* is assigned as *B. thermacidophilum* subsp. *thermacidophilum*.

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

Taxa: 1, *B. thermacidophilum* subsp. *porcinum* subsp. nov. P3-14^T; 2, *B. thermacidophilum* AS 1.2282^T; 3, *B. thermophilum* JCM 1207^T; 4, *B. boum* JCM 1211^T [data from Scardovi *et al.* (1979)]. +, Positive reaction; –, negative reaction; w, weak reaction; D, variable reaction.

Characteristic	1	2	3	4
Fermentation of:				
L-Arabinose	–	+	–	–
D-Mannose	–	+	–	–
Methyl α-D-glucoside	w	+	w	
Inulin	–	+	w	D
Trehalose	+	–	–	D
Maximum growth temp (°C)	46.5	49.5	47	45
Minimum growth pH	4.0	4.0	4.2	>5.0
DNA G + C content (mol%)	61.5	57.7	60	60

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, gluconate, inulin, lactose, D-mannose, methyl α-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, 36^T (= AS 1.2282^T = LMG 21395^T), 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, P3-14^T (= AS 1.3009^T = LMG 21689^T), was isolated from the faeces of a piglet.

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References

- Biavati, B. & Mattarelli, P. (1991). *Bifidobacterium ruminantium* sp. nov. and *Bifidobacterium merycicum* sp. nov. from the rumens of cattle. *Int J Syst Bacteriol* **41**, 163–168.
- Biavati, B., Mattarelli, P. & Crociani, F. (1991). *Bifidobacterium saeculare*: a new species isolated from feces of rabbit. *Syst Appl Microbiol* **14**, 389–392.
- Cai, Y., Matsumoto, M. & Benno, Y. (2000). *Bifidobacterium lactis* Meile *et al.* 1997 is a subjective synonym of *Bifidobacterium animalis* (Mitsuoka 1969) Scardovi and Trovatelli 1974. *Microbiol Immunol* **44**, 815–820.
- Crociani, F., Biavati, B., Alessandrini, A., Chiarini, C. & Scardovi, V. (1996). *Bifidobacterium inopinatum* sp. nov. and *Bifidobacterium denticolens* sp. nov., two new species isolated from human dental caries. *Int J Syst Bacteriol* **46**, 564–571.
- Dong, X., Xin, Y., Jian, W., Liu, X. & Ling, D. (2000). *Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester. *Int J Syst Evol Microbiol* **50**, 119–125.
- Hoyle, L., Inganäs, E., Falsen, E., Drancourt, M., Weiss, N., McCartney, L. A. & Collins, M. D. (2002). *Bifidobacterium scardovii* sp. nov., from human sources. *Int J Syst Evol Microbiol* **52**, 995–999.
- Jian, W. & Dong, X. (2002). Transfer of *Bifidobacterium inopinatum* and *Bifidobacterium denticolens* to *Scardovia inopinata* gen. nov., comb. nov., and *Parascardovia denticolens* gen. nov., comb. nov., respectively. *Int J Syst Evol Microbiol* **52**, 809–812.
- Jian, W., Zhu, L. & Dong, X. (2001). New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. *Int J Syst Evol Microbiol* **51**, 1633–1638.
- Kwok, A. Y. C., Su, S.-C., Reynolds, R. P., Bay, S. J., Av-Gay, Y., Dovichi, N. J. & Chow, A. W. (1999). Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus *Staphylococcus*. *Int J Syst Bacteriol* **49**, 1181–1192.
- Lauer, E. (1990). *Bifidobacterium gallicum* sp. nov. isolated from human feces. *Int J Syst Bacteriol* **40**, 100–102.
- Lauer, E. & Kandler, O. (1983). DNA-DNA homology, murein types and enzyme patterns in the type strains of the genus *Bifidobacterium*. *Syst Appl Microbiol* **4**, 42–64.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Marston, E. L., Sumner, J. W. & Regnery, R. L. (1999). Evaluation of intraspecies genetic variation within the 60 kDa heat-shock protein gene (*groEL*) of *Bartonella* species. *Int J Syst Bacteriol* **49**, 1015–1023.
- Meile, L., Ludwig, W., Rueger, U., Gut, C., Kaufmann, P., Dasen, G., Wenger, S. & Teuber, M. (1997). *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species isolated from fermented milk. *Syst Appl Microbiol* **20**, 57–64.
- Miyake, T., Watanabe, K., Watanabe, T. & Oyaizu, H. (1998). Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. *Microbiol Immunol* **42**, 661–667.
- Olsen, G. J., Woese, C. R. & Overbeek, R. (1994). The winds of (evolutionary) change: breathing new life into microbiology. *J Bacteriol* **176**, 1–6.
- Requena, T., Burton, J., Matsuki, T., Munro, K., Simon, M. A., Tanaka, R., Watanabe, K. & Tannock, G. W. (2002). Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene. *Appl Environ Microbiol* **68**, 2420–2427.
- Roy, D. & Ward, P. (1990). Evaluation of rapid methods for differentiation of *Bifidobacterium* species. *J Appl Bacteriol* **69**, 738–749.
- Sakata, S., Kitahara, M., Sakamoto, M., Hayashi, H., Fukuyama, M. & Benno, Y. (2002). Unification of *Bifidobacterium infantis* and *Bifidobacterium suis* as *Bifidobacterium longum*. *Int J Syst Evol Microbiol* **52**, 1945–1951.
- Scardovi, V. (1986). Genus *Bifidobacterium* Orla-Jensen. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1418–1434. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Scardovi, V., Trovatelli, L. D., Biavati, B. & Zani, G. (1979). *Bifidobacterium cuniculi*, *Bifidobacterium choerinum*, *Bifidobacterium boum*, and *Bifidobacterium pseudocatenulatum*: four new species and their deoxyribonucleic acid homology relationships. *Int J Syst Bacteriol* **29**, 291–311.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.