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Subspeciation of *Bifidobacterium longum* by multilocus approaches and amplified fragment length polymorphism: Description of *B. longum* subsp. *suillum* subsp. nov., isolated from the faeces of piglets☆

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ABSTRACT

The species *Bifidobacterium longum* is currently divided into three subspecies, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. longum* subsp. *suis*. This classification was based on an assessment of accumulated information on the species' phenotypic and genotypic features. The three subspecies of *B. longum* were investigated using genotypic identification [amplified-fragment length polymorphism (AFLP), multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST)]. By using the AFLP and the MLSA methods, we allocated 25 strains of *B. longum* into three major clusters corresponding to the three subspecies; the cluster comprising the strains of *B. longum* subsp. *suis* was further divided into two subclusters differentiable by the ability to produce urease. By using the MLST method, the 25 strains of *B. longum* were divided into eight groups: four major groups corresponding to the results obtained by AFLP and MLSA, plus four minor disparate groups. The results of AFLP, MLSA and MLST analyses were consistent and revealed a novel subspeciation of *B. longum*, which comprised three known subspecies and a novel subspecies of urease-negative *B. longum*, for which the name *B. longum* subsp. *suillum* subsp. nov. is proposed, with type strain Su 851^T = DSM 28597^T = JCM 19995^T.

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Introduction

Currently, the names of 44 species and nine subspecies of the genus *Bifidobacterium* have been validly published (http://www.bacterio.net/bifidobacterium.html). Among these species, *Bifidobacterium longum* is considered one of the most important contributors to host health and representative strains are frequently used as a probiotics [8,12,58]. *B. longum* was described by Reuter [36] using a strain isolated from adult faeces. Later the species was recognised as one of the most prevalent bifidobacterial species in the gastrointestinal tract of human adults [2]. *Bifidobacterium infantis* was also proposed by Reuter [36] using strains that were prevalent in the gastrointestinal tract of infants [3,26] while *Bifidobacterium suis* was described by Matteuzzi et al. [29] for strains isolated from pig faeces.

In surveys of DNA–DNA relatedness, *B. infantis*, *B. longum* and *B. suis* have been shown to have hybridisation rates of about 70% and higher [19,41], while possessing more than 97% 16S rRNA gene sequence identity [32], suggesting interrelationships at the species level [46]. In addition, these species showed high average nucleotide identity (ANI) values ranging from 95.5 to 96.6%, which were higher than 95% cut-off value recommended for species demarcation [16,21]. Sakata et al. [39] unified *B. longum, B. infantis* and *B. suis* into the single species, *B. longum*, on the basis of DNA–DNA hybridisation values, ribotyping and RAPD-PCR. They

☆ The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB924514–AB924536 (16S rRNA), AB916466–AB916490 (*clpC*), AB917469–AB917493 (*dnaG*), AB917494–AB917518 (*dnaJ1*), AB917519–AB917543 (*hsp60*), AB924075–AB924081 (*purF*), AB917544–AB917568 (*rpoC*) and AB917569–AB917593 (*xfp*).

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established three biotypes – 'longum', 'infantis' and 'suis' – for strains belonging to the respective former species. However, a variety of other genotypic techniques, including transaldolase-specific PCR-denaturing gradient gel electrophoresis [35], comparison of the *recA*, *tuf* and *ldh* gene sequences [37,51] and repetitive element sequence-based PCR fingerprinting [25,52], as well as the results of PAGE experiments on soluble proteins [1], clearly allowed to discriminate these three species. Mattarelli et al. [27] assessed the published results in order to resolve the taxonomic dispute. In agreement with the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Bifidobacterium*, *Lactobacillus* and related organisms, they have proposed that these three species should be reclassified into three subspecies, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. longum* subsp. *suis*. Given the wide applicability of the species, there is a strong need for reliable identification of strains of *Bifidobacterium* at species or subspecies level, improving studies of their ecological distribution and biological features.

The amplified-fragment length polymorphism (AFLP) method has been used to discriminate strains so as to verify the mother-to-infant transmission of *B. longum* subsp. *longum* [23]. Here, we chose to use AFLP, regarded as a reliable nucleic acid fingerprinting method [50] was used to investigate the subspecies of *B. longum* because of the success of this technique in classifying subspecies of *Lactobacillus delbrueckii* [48].

In addition, the multilocus sequence analysis or -typing (MLSA or MLST) method has been widely used to discriminate bacterial strains [23,48]. MLSA is based on the use of a phylogenetic analysis of nucleotide sequences of housekeeping genes of strains belonging to closely related species, allowing to determine phylogenetic clustering patterns. Concatenation of genes was shown to be extremely useful for precise bacterial phylogenetic analysis [49]. Ventura et al. [53] used seven housekeeping-gene sequences – of *clpC*, *dnaB*, *dnaG*, *dnaJ1*, *purF*, *rpoC* and *xfp* – to analyse the phylogenetic relationships of *Bifidobacterium* species; they recommended the use of a phylogeny based upon concatenated sequences to improve the identification of members of the *Bifidobacterium* at species level. Delétoile et al. [7] also confirmed that phylogenetic analysis based on the concatenated sequences of seven housekeeping genes (*clpC*, *fusA*, *gyrB*, *ileS*, *purF*, *rplB* and *rpoB*) indicated a distinct separation of *B. longum* subsp. *infantis* from the cluster composed of subspecies' *longum* and *suis*. MLST is used to analyse intraspecific diversity by comparing allelic profiles [22]. A novel subspeciation of *L. delbrueckii* was revealed by using MLST based on the analysis of seven housekeeping genes (*fusA, gyrB, hsp60, ileS, pyrG, recA* and *recG*) [48]. By using MLST based on seven housekeeping genes (*clpC*, *dnaG*, *dnaJ*, *fusA*, *gyrB*, *purF* and *rpoB*), Makino et al. [23] revealed the transmission of intestinal *B. longum* subsp. *longum* strains from mother to infant. The results obtained by using the MLST method corresponded well to the results of AFLP with respect to the identification of 207 *B. longum* subsp. *longum* strains. It is very likely that continued whole genome sequencing results will assist in better quantifying and clarifying taxonomic and functional differences between strains of *B. longum*.

The aim of the present study was to: (i) further examine the subspecies composition of *B. longum* using the above listed methodologies and (ii) adapt the current taxonomic structure of the species if shown necessary.

Materials and methods

Bacterial strains and growth conditions

A total of 25 bacterial strains assigned as *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* or *B. longum* subsp. *suis* were obtained from the Culture Collection of the Yakult Central Institute (YIT; Tokyo, Japan) and the Bologna University Scardovi Collection of Bifidobacteria (Su; BUSCoB Bologna, Italy) (Table 1). All bacterial strains were grown at 37 °C for 24 h in GAM broth (Nissui, Tokyo, Japan) supplemented with 0.5% glucose.

Phenotypic characterisation

Morphological, cultural and biochemical testing according to standard techniques was performed at 37 °C unless otherwise stated. Gram staining and catalase activity were determined

Table 1
Bacterial strains used in this study.

Species/subspecies	Strain	Additional strain information	Source	Notes
B. longum subsp. *longum*	YIT 4021^T	ATCC 15707^T	Intestine of human adult	
	YIT 4037	ATCC 15708	Intestine of human infant	
	YIT 10936		Faeces of human infant	
	YIT 10937		Faeces of human infant	
	YIT 10938		Faeces of human infant	
	YIT 11061		Intestine of human adult	
	YIT 11074		Faeces of adult human	
	YIT 11976	ATCC 55813	Faeces of human infant	
	YIT 11977	ATCC 55814	Faeces of human infant	
	YIT 12147		Intestine of human adult	
	YIT 12736	DSM 20097	Faeces of calf	
B. longum subsp. *infantis*	YIT 4019	ATCC 15702	Intestine of human infant	
	YIT 4020	ATCC 25962	Intestine of human infant	
	YIT 4081		Faeces of human infant	
	YIT 11889		Faeces of human infant	
	YIT 11945		Faeces of human infant	
	YIT 12734^T	DSM 20088^T	Intestine of human infant	
	YIT 12735	DSM 20218	Intestine of human infant	
B. longum subsp. *suis*	YIT 4082^T	JCM 1269^T, Su 859	Faeces of piglet	urease positive
	YIT 4108	JCM 7139, Su 901	Faeces of piglet	urease positive
	Su 868		Faeces of piglet	urease positive
	Su 903		Faeces of piglet	urease positive
	Su 923		Faeces of piglet	urease positive
B. longum subsp. *suillum* subsp. nov.	Su 851^T		Faeces of piglet	urease negative
	Su 864		Faeces of piglet	urease negative

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