Sharpea azabuensis gen. nov., sp. nov., a Gram-positive, strictly anaerobic bacterium isolated from the faeces of thoroughbred horses

Hidetoshi Morita,¹ Chiharu Shiratori,¹ Masaru Murakami,¹ Hideto Takami,² Hidehiro Toh,³ Yukio Kato,¹ Fumihiko Nakajima,⁴ Misako Takagi,⁵ Hiroaki Akita,⁴ Toshio Masaoka¹ and Masahira Hattori⁶

Correspondence

Hidetoshi Morita

morita@azabu-u.ac.jp

¹School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagami-hara, Kanagawa 229-8501, Japan

²Microbial Genome Research Group, Japan Agency of Marine-Earth Science and Technology, 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan

³RIKEN Genomic Sciences Center, 1-7-22 Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

⁴Northern Farm, 275 Hayakita-genbu, Abira-cho, Yufutsu-gun, Hokkaido 059-1432, Japan

⁵Clossfield-Bio Inc., 1-1-20 Higashi-nihonbashi, Chuo-ku, Tokyo 103-0004, Japan

⁶Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

Four bacterial strains, designated ST18^T, HM244, HM250 and DI49, were isolated from the fresh faeces of four thoroughbred horses in Japan. Cells were Gram-positive, strictly anaerobic, catalase-negative, non-spore-forming, non-motile rods that occurred in chains. They were placed in the same subcluster based on 16S rRNA gene sequence analysis, phenotypic characteristics and levels of DNA–DNA relatedness. Their DNA G + C content ranged from 36 to 38 mol%. *Lactobacillus catenaformis*, *Lactobacillus vitulinus* and *Catenibacterium mitsuokai* belong to cluster XVII of the *Clostridium* subphylum. Strain ST18^T was most closely related to *L. catenaformis* ATCC 25536^T in the phylogenetic tree, but these strains shared only 89.9 % (1336/1486 bp) 16S rRNA gene sequence similarity. *L. catenaformis*, *L. vitulinus* and *C. mitsuokai* are homofermentative bacteria, whereas ST18^T produced CO₂ from glucose. Whereas the cell-wall peptidoglycan type of *L. catenaformis* and *L. vitulinus* was L-Lys–L-Ala₃, that of *C. mitsuokai* and the subgroup represented by ST18^T was A1_γ (L-Ala–D-Glu–*meso*-diaminopimelic acid). On the basis of 16S rRNA gene sequence divergence of more than 10 % from *L. catenaformis* as well as phenotypic characteristics, strains ST18^T, HM244, HM250 and DI49 are considered to represent a novel species of a new genus belonging to the *Clostridium* subphylum cluster XVII, for which the name *Sharpea azabuensis* gen. nov., sp. nov. is proposed. The type strain of *Sharpea azabuensis* is ST18^T (=JCM 14210^T =DSM 18934^T).

Various Gram-positive, non-spore-forming, rod-shaped anaerobic bacteria belonging to the *Clostridium* subphylum

Abbreviation: *m*-Dpm, *meso*-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ST18^T, HM244, HM250 and DI49 and *Lactobacillus vitulinus* JCM 1143^T are AB210824, AB360599, AB360600, AB360601 and AB210825, respectively.

Maximum-parsimony and maximum-likelihood phylogenetic trees and tables detailing the sources of strain isolation and levels of DNA–DNA relatedness are available as supplementary material with the online version of this paper.

make up the predominant intestinal microflora in mammals. Some genera of the *Clostridium* subphylum have been inadequately described (Collins *et al.*, 1994; Kageyama & Benno, 2000a, b; Willems & Collins, 1996; Willems *et al.*, 1996) and, because of their broad definitions, these genera have acted as a depository for a large number of phylogenetically diverse species. For example, some species originally assigned to the genus *Eubacterium* in the *Clostridium* subphylum have subsequently been transferred to other genera or to new genera (Kageyama *et al.*, 1999a, b; Ludwig *et al.*, 1992; Nakazawa *et al.*, 2000; Taras *et al.*, 2002; Wade *et al.*, 1999; Willems

& Collins, 1996). The clusters of the *Clostridium* subphylum provide a useful starting point for investigations of phylogenetic relationships among the Gram-positive, non-spore-forming, rod-shaped anaerobic bacteria of the mammalian intestinal tract. The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*, and its closest relatives, grouped within the same family, are members of the genera *Paralactobacillus* and *Pediococcus* (Garrity *et al.*, 2007). *Lactobacillus vitulinus* and *Lactobacillus cateniformis* are related only distantly to other recognized *Lactobacillus* species (Hammes & Vogel, 1995; Hammes & Hertel, 2006; Pot *et al.*, 1994; Schleifer & Ludwig, 1996), given that they belong to cluster XVII of the *Clostridium* subphylum rather than to the *Lactobacillales* (Collins *et al.*, 1994).

In the present study, four bacterial strains (designated ST18^T, HM244, HM250 and DI49) were isolated from the faeces of four thoroughbred horses. A polyphasic taxonomic study of these strains was performed based on phenotypic characterization and phylogenetic as well as genetic methods. The results obtained suggest that these four strains represent a single novel species of a new genus belonging to cluster XVII of the *Clostridium* subphylum.

Fresh faeces of four thoroughbred horses were collected from two stables and one farm in Japan (for details see Supplementary Table S1 in IJSEM Online). The faecal specimens were transferred to the laboratory within 24 h under anaerobic conditions by use of an AnaeroPak (Mitsubishi Gas Chemical) at 4 °C. A diluent containing 0.85 % NaCl, 0.05 % sodium pyruvate and 0.05 % sodium ascorbate was used. Dissolved oxygen was removed by bubbling the solution with nitrogen gas for 60 min. Initial processing and subsequent weighing and dilution of the specimens were carried out under anaerobic conditions. Each dilution was then spread on blood liver (BL) agar plates (Eiken Chemical Co.) containing 0.05 % sodium pyruvate and 0.05 % sodium ascorbate and incubated anaerobically at 37 °C for 2 days. All further cultivation was performed at 37 °C in anaerobic bacterial culture medium (ABCM) broth (Eiken Chemical Co.). *L. cateniformis* JCM 1121^T, *L. vitulinus* JCM 1143^T and *Catenibacterium mitsuokai* JCM 10609^T, belonging to the *Clostridium* subphylum cluster XVII, were obtained from the Japan Collection of Microorganisms (JCM).

Approximately 1500 bp of the 16S rRNA gene sequences of 137 isolates were determined by using standard primers (27F, 520F, 930F, 1100F, 75R, 520R, 800R and 1100R) (Weisburg *et al.*, 1991; Miyake *et al.*, 1998) via an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). More than 99 % 16S rRNA gene sequence similarity was observed (approximately 1500 bp) among strains ST18^T, HM244, HM250 and DI49 (see Supplementary Table S1 for accession numbers). The G+C content of the DNA was determined by enzymically hydrolysing the DNA and quantifying the nucleosides by using HPLC based on the

method proposed by Ezaki *et al.* (1990). The DNA G+C contents of the four novel strains ranged from 36.8 to 37.9 mol%. Fluorometric DNA–DNA hybridization was carried out in microdilution wells according to the method proposed by Ezaki *et al.* (1989). DNA–DNA hybridization analyses were performed among the four strains, and levels of relatedness between strain ST18^T and strains HM244, HM250 and DI49 were 81.8, 93.1 and 89.2 %, respectively. These values are above the 70 % cut-off recommended for the delineation of separate bacterial species (Stackebrandt & Goebel, 1994), indicating that the four novel strains represented the same genomic species.

The closest known relatives of the isolates were determined by performing sequence similarity database researches. Multiple alignments of the sequences were carried out by using CLUSTAL W (Thompson *et al.*, 1994). The robustness of individual branches was estimated by using a bootstrap analysis of 1000 replicates (Felsenstein, 1985). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods within PHYLIP version 3.66 (Felsenstein, 2005). In the neighbour-joining dendrogram created based on 16S rRNA gene sequences, strain ST18^T was placed within the unique *L. vitulinus* phylogenetic group (Felis & Dellaglio, 2007) and was related closely to *L. cateniformis*, *L. vitulinus* and *Catenibacterium mitsuokai* (Fig. 1). Similar phylogenetic trees were obtained with the maximum-likelihood and maximum-parsimony methods (Supplementary Figs S1 and S2). The 16S rRNA gene sequence of strain ST18^T showed 89.9 % (1336/1486 bp) similarity to that of *L. cateniformis* ATCC 25536^T. The physiological characteristics of strain ST18^T were thus compared with those of *L. cateniformis* JCM 1121^T, *L. vitulinus* JCM 1143^T and *Catenibacterium mitsuokai* JCM 10609^T (Table 1).

The DNA G+C content of strain ST18^T was 37.4 mol%, higher than the values obtained for *L. cateniformis* JCM 1121^T (34.8 mol%), *L. vitulinus* JCM 1143^T (34.4 mol%) and *Catenibacterium mitsuokai* JCM 10609^T (36.6 mol%). Carbohydrate fermentation patterns were determined by using a test medium that comprised 10 g peptone (Becton, Dickinson; BD), 10 g tryptone (BD), 3 g soy peptone (BD), 2.5 g potassium dihydrogen phosphate (Nacalai tesque), 3 g meat extract (BD), 2 g NaCl (Nacalai tesque), 0.3 g L-cysteine hydrochloride (Nacalai tesque), 0.005 g haemin (Nacalai tesque), 0.3 g sodium thioglycolate (Nacalai tesque), 0.17 g bromocresol purple (Nacalai tesque), and glucose, galactose, fructose, mannose, cellobiose, lactose, salicin, melibiose, trehalose, raffinose, starch, D-arabinose, D-ribose, D-xylose, rhamnose, mannitol, sorbitol or melezitose (all at 10 g l⁻¹; Nacalai tesque) as carbohydrates at pH 6.8. The results were recorded after 48 h at 37 °C under anaerobic conditions. Strain ST18^T fermented and produced acid from glucose, galactose, fructose, mannose, cellobiose, lactose, melibiose and starch. However, as some of the novel strains were able to ferment

Fig. 1. Phylogenetic relationships between strains ST18^T, HM244, HM250 and DI49 and species in clusters XV–XIX of the *Clostridium* subphylum based on 16S rRNA gene sequences. The tree was constructed by using the neighbour-joining method. Bootstrap values above 70% are indicated at branch points. *Eubacterium limosum* ATCC 8486^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

salicin, trehalose and raffinose, it was difficult to distinguish this subcluster from *L. cateniformis*, *L. vitulinus* and *Catenibacterium mitsuokai* based on carbohydrate fermentation patterns (Table 1). The isomer of lactic acid produced from glucose was determined by using a

D/L-lactic acid F-kit (Roche Diagnostics). Strain ST18^T and the three other faecal strains produced only D(–)-lactic acid. Other biochemical tests, such as those relating to motility, growth at a fixed temperature and gas production from glucose, were performed by using the methods

Table 1. Physiological characteristics of strains ST18^T, HM244, HM250 and DI49 and related type strains

Reference strains: 1, *L. cateniformis* JCM 1121^T (data from this study); 2, *L. vitulinus* JCM 1143^T (data from Sharpe *et al.*, 1973); 3, *Catenibacterium mitsuokai* JCM 10609^T (data from Kageyama & Benno, 2000a). +, Positive; –, negative; w, weakly positive; ND, no data available. All strains were positive for the fermentation of glucose, galactose, fructose, mannose, cellobiose and lactose. All strains were negative for aerobic growth, growth at 15 °C, catalase activity and fermentation of rhamnose, mannitol, sorbitol and melezitose. DNA G+C contents were determined by HPLC.

Characteristic	ST18 ^T	HM244	HM250	DI49	1	2	3
Lactic acid isomer(s)	D	D	D	D	D	D	DL
Gas production from glucose	+	+	+	+	–	–	–
Fermentation style*	HE	HE	HE	HE	HO	HO	HO
Fermentation of:							
Salicin	–	+	+	–	+	+	+
Melibiose	+	+	+	+	–	+	ND
Trehalose	–	+	+	–	–	–	–
Raffinose	–	+	+	+	–	–	–
Starch	+	w	+	+	+	+	–
D-Xylose	–	–	–	–	+	–	–
D-Arabinose	–	–	–	–	+	–	–
D-Ribose	–	–	–	–	+	–	–
DNA G+C content (mol%)	37.4	37.9	36.8	37.4	34.8	34.4	36.6
Peptidoglycan type	A1γ (L-Ala–D-Glu– <i>m</i> -Dpm)	A1γ (L-Ala–D-Glu– <i>m</i> -Dpm)	A1γ (L-Ala–D-Glu– <i>m</i> -Dpm)	A1γ (L-Ala–D-Glu– <i>m</i> -Dpm)	L-Lys–L-Ala ₃	L-Lys–L-Ala ₃	A1γ (L-Ala–D-Glu– <i>m</i> -Dpm)

*HE, Heterofermentative; HO, homofermentative.

described by Mitsuoka (1969). Table 1 details the characteristics most useful in distinguishing the strains studied from closely related bacteria. As strain ST18^T and the other three novel strains produced gas (CO₂) from glucose, this phenomenon clearly distinguished them from *L. catenaformis*, *L. vitulinus* and *Catenibacterium mitsuokai*.

DNA–DNA hybridization analyses were performed between strain ST18^T, *L. catenaformis* JCM 1121^T, *L. vitulinus* JCM 1143^T and *Catenibacterium mitsuokai* JCM 10609^T. Levels of relatedness were less than the 70 % cut-off point recommended for the delineation of species (Stackebrandt & Goebel, 1994) (Supplementary Table S2). Based on the methods proposed by Komagata & Suzuki (1987), meso-diaminopimelic acid (*m*-Dpm) was found in the peptidoglycan layer of the subgroup represented by strain ST18^T by using HPLC. The cell-wall peptidoglycan of strain ST18^T contained *m*-Dpm, glutamic acid and alanine at a molar ratio of 1:1:2, as determined by ultrahigh-performance liquid chromatography. The structural type was A1γ (L-Ala–D-Glu–*m*-Dpm). Strains HM244, HM250 and DI49 possessed the same peptidoglycan type (as did *Catenibacterium mitsuokai*). In contrast, the cell-wall peptidoglycan type of *L. catenaformis* and *L. vitulinus* was L-Lys–L-Ala₃.

The four novel strains isolated from the faeces of thoroughbred horses clearly belonged to a hitherto unrecognized, Gram-positive anaerobic species within the *Clostridium* subphylum cluster XVII. Based on 16S rRNA gene sequence comparisons, the bacterium has a close phylogenetic relationship with *L. catenaformis*, *L. vitulinus* and *Catenibacterium mitsuokai*. Although the association between these taxa was significant (Supplementary Table S1), a 16S rRNA gene sequence divergence of more than 10 % suggested that this relationship was one of phylogenetically closely related but different genera. On the basis of 16S rRNA gene sequence considerations and other phenotypic characteristics, these strains merit classification within a novel species of a new genus, for which the name *Sharpea azabuensis* gen. nov., sp. nov. is proposed.

Description of *Sharpea* gen. nov.

Sharpea (Shar.pe'a. N.L. fem. n. *Sharpea* named in honour of Michaela E. Sharpe, for her considerable efforts in the development of evolutionary microbiology).

Cells occur in short chains and spores are absent. Gram-positive, strictly anaerobic and heterofermentative bacteria. The cell wall contains *m*-Dpm, glutamic acid and alanine, and the peptidoglycan type is A1γ (L-Ala–D-Glu–*m*-Dpm). The G + C content of the DNA of known strains is 36.8–37.9 mol%. The genus *Sharpea* is a member of cluster XVII of the *Clostridium* subphylum of Gram-positive bacteria and exhibits a close phylogenetic association with *Lactobacillus catenaformis*, *Lactobacillus vitulinus* and *Catenibacterium mitsuokai*. The type species is *Sharpea azabuensis*.

Description of *Sharpea azabuensis* sp. nov.

Sharpea azabuensis (a.za.bu.en'sis. N.L. fem. adj. *azabuensis* arbitrary name referring to the fact that the study in which the species was described was supported by the Azabu 'Academic Frontier' Project for Private Universities: Matching Fund Subsidy).

Cells are catalase-negative, non-motile rods (2–10 μm long and 0.7–1.0 μm wide). When grown on BL agar at 37 °C for 24 h, colonies are 1.0–2.5 mm, circular to slightly irregular, with undulating edges, flat in elevation, smooth, brown, transparent and butyrous. The optimum growth temperature is 37 °C. Unable to grow under aerobic conditions, in the presence of 4.5 % NaCl or at 15 °C, but grows in the presence of 3 % NaCl and at 45 °C. Metabolizes glucose heterofermentatively, and produces D(–)-lactic acid and CO₂. Acids are produced by the type strain from glucose, galactose, fructose, mannose, cellobiose, lactose, melibiose and starch. Some strains can ferment salicin, trehalose and raffinose. Unable to ferment rhamnose, mannitol, sorbitol or melezitose.

The type strain, ST18^T (=JCM 14210^T =DSM 18934^T), was isolated from the faeces of a thoroughbred horse. HM244, HM250 and DI49, also isolated from faeces of thoroughbred horses, are additional strains of the species.

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