Selenomonas bovis sp. nov., isolated from yak rumen contents

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Two strictly anaerobic, crescent-shaped bacterial strains, designated WG^T and Ycb08, were isolated from a cellulose-degrading mixed culture enriched from yak rumen contents. The strains were Gram-negative, non-spore-forming and motile, with four to six flagella situated at the centre of the concave side of the cell. The cells were 0.9–1.1×4–6 µm. Growth was observed at 27–46 °C (optimum 39 °C) and pH 4.2–8.3 (optimum pH 7.0–7.2). Arabinose, glucose, mannose, cellobiose, lactose, sucrose, trehalose, melibiose, raffinose, salicin and aesculin were fermented. The end products of glucose fermentation were acetate, propionate and CO₂. The G+C contents of strains WG^T and Ycb08 were respectively 63.9±0.2 and 62.5±0.2 mol% (*T_m*). Phylogenetic analysis based on 16S rRNA gene sequences revealed that the two strains were related to the genera *Mitsuokella* and *Selenomonas* at similarity levels below 97 %; however, they differed from members of the genus *Mitsuokella* in their flagellar arrangement. On the basis of phenotypic, genotypic and physiological evidence, strains WG^T and Ycb08 are identified as members of a novel species of the genus *Selenomonas*, for which the name *Selenomonas bovis* sp. nov. is proposed. The type strain is WG^T (=CGMCC 1.5073^T =JCM 15470^T).

The yak (*Bos grunniens*) is a ruminant found mainly on the Qinghai–Tibet Plateau, China, at altitudes over 3000 m above sea level, with grasses as their main food. Previous work in our laboratory (An *et al.*, 2005) based on a culture-independent approach determined that more than 60 % of the bacteria in the yak rumen clustered in not-yet-cultured groups, displaying <90 % 16S rRNA gene sequence similarity with cultured species. In addition, a relatively large proportion of the species detected in the yak rumen grouped with fibrolytic bacteria isolated previously from rumen contents, suggesting that the yak rumen could harbour novel types of fibrolytic bacteria. Therefore, by using filter paper as the sole carbon source, we obtained a cellulose-degrading mixed-culture enrichment with yak rumen content as inoculant; a few fibrolytic and non-fibrolytic bacterial strains were isolated from the mixed culture. In this study, we describe two non-fibrolytic bacteria strains that represent a novel species of the genus *Selenomonas*.

Yak rumen content was inoculated into a modified basal medium (Hungate, 1966) with filter paper as the sole carbon source and grown under a gas phase of N₂/CO₂

(80:20). The medium contained 20 % rumen fluid and 80 % basal medium. A fibrolytic mixed culture was obtained by subculturing the enrichment in the same medium for eight to ten transfers. After serial dilution in peptone-yeast extract-glucose (PYG) broth (Holdeman *et al.*, 1977) and the use of the Hungate roll-tube technique (Hungate, 1969), single colonies were observed after incubation at 39 °C for 2 days. Colonies were picked and transferred to the same medium. The roll-tube procedure was repeated several times before strains WG^T and Ycb08 were obtained. The purity of the isolates was examined by light microscopy. All inoculations and transfers were done with syringes and needles, and the cultures were incubated at 39 °C unless indicated. *Selenomonas ruminantium* subsp. *ruminantium* DSM 2150^T, *S. ruminantium* subsp. *lactilytica* DSM 2872^T, *Selenomonas sputigena* DSM 20758^T and *Mitsuokella jalaludinii* DSM 13811^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and served as reference strains in physiological characterization tests.

Cell morphology was examined under a light microscope (Olympus BH-2) and an electron microscope (Hitachi H-600A). For electron microscopy studies, bacterial cells were negatively stained with uranyl acetate. Spore formation was examined by phase-contrast microscopy at the end of growth as well as by heat treatment in a water bath at 80 °C for 10 min.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WG^T and Ycb08 are respectively EF139191 and EU821596.

A transmission electron micrograph of a cell of strain WG^T is available as supplementary material with the online version of this paper.

Cells were Gram-negative, typical curved rods, $0.9\text{--}1.1 \times 4\text{--}6\text{ }\mu\text{m}$, and occurred singly or in pairs. Spores were never observed, and the strains could not survive heat treatment at $80\text{ }^{\circ}\text{C}$ for 10 min. Negatively stained cells revealed the presence of four to six flagella in a tuft situated near the centre of the concave side (Supplementary Fig. S1, available in IJSEM Online).

Growth was determined by monitoring the OD_{600} of the culture in PYG medium at $39\text{ }^{\circ}\text{C}$. The strains were strictly anaerobic; no growth occurred when exposed to air. The generation time of strain WG^{T} was determined as 2.3 h by monitoring the OD_{600} at 30 min intervals for 24 h. The temperature profile of strain WG^{T} was determined in a water bath from 20 to $50\text{ }^{\circ}\text{C}$ at $1\text{ }^{\circ}\text{C}$ intervals (pH 7.0) and growth was observed between 27 and $46\text{ }^{\circ}\text{C}$, with optimal growth at $39\text{ }^{\circ}\text{C}$. The pH range for growth was pH 4.2–8.3, with optimal growth at around pH 7.0–7.2, as determined in PYG broth at various pH values adjusted with Na_2HPO_4 and NaH_2PO_4 .

The end products from glucose fermentation were determined by growing strains WG^{T} and Ycb08 in PYG medium; short-chain fatty acids were then analysed by using a gas chromatograph (GC-14B; Shimadzu) equipped with a flame-ionization detector (column temperature $230\text{ }^{\circ}\text{C}$, injector $250\text{ }^{\circ}\text{C}$, detector $280\text{ }^{\circ}\text{C}$). The carrier gas was N_2 at a flow rate of 30 ml min^{-1} . It was determined that strain WG^{T} and Ycb08 produced acetate, propionate and CO_2 from glucose at a molar ratio of 0.7:1.1:1.2.

Substrate utilization was tested by growing the two strains in PY medium (Holdeman *et al.*, 1977) separately containing each of the following substrates: lactate, glycerol, dulcitol, mannitol, sorbitol, xylose, arabinose, glucose, mannose, cellobiose, lactose, sucrose, trehalose, melibiose, raffinose, inulin, salicin and aesculin. Stock solutions of the test compounds were prepared anaerobically, sterilized by filtration and injected into PY medium to the final concentration of 1% (w/v). Other physiological characteristics including the Voges–Proskauer test, nitrate reduction, indole production, H_2S production, methyl red test and gelatin liquefaction were determined according to Holdeman *et al.* (1977).

The two strains exhibited almost identical physiological and biochemical profiles except that strain Ycb08 could not ferment inulin or raffinose. Other physiological characteristics are listed in the species description.

Genomic DNA was extracted and purified according to Marmur (1961). The G+C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962) using a DU800 spectrophotometer (Beckman) with *Escherichia coli* K-12 DNA as the reference. The G+C contents of the genomic DNA for strains WG^{T} and Ycb08 were determined as 63.9 ± 0.2 and 62.5 ± 0.2 mol%, respectively, from three parallel measurements.

The 16S rRNA gene was amplified using the bacterial universal primers 27F and 1541R (Weisburg *et al.*, 1991).

Purified PCR products approximately 1.5 kbp in length were cloned to *Escherichia coli* DH5 α and sequenced by the Sangon Biological Engineering Technology Service (Shanghai, China). The sequences were submitted to GenBank to search for similar sequences using BLAST algorithm. The 16S rRNA gene sequences of strain WG^{T} and reference strains retrieved from GenBank were aligned using the CLUSTAL_X program v. 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the neighbour-joining, UPGMA, minimum evolution and maximum-parsimony methods as implemented in MEGA3 (Kumar *et al.*, 2004). Stability of groupings was evaluated by bootstrap analysis of 1000 datasets (Felsenstein, 1985). Strains WG^{T} and Ycb08 displayed 16S rRNA gene sequence similarity of 99.7%; they were therefore considered to represent a single species. To ascertain the phylogenetic position of the new isolates, the complete 16S rRNA gene sequences were compared with the most similar sequences retrieved from GenBank. On the basis of a consensus 1379 bp 16S rRNA gene sequence, a phylogenetic tree rooted with *Bacillus subtilis* DSM 10 $^{\text{T}}$ was constructed (Fig. 1). It was shown that the two strains clustered with the members of the genera *Selenomonas* and *Mitsuokella* and were closest to *M. jalaludinii* M 9 $^{\text{T}}$ (95.5% 16S rRNA gene sequence similarity), followed by *S. ruminantium* subsp. *ruminantium* GA192 $^{\text{T}}$ (95.4%), *S. ruminantium* subsp. *lactilytica* HD $_4$ (95.2%) and *Mitsuokella multacida* NCTC 10934 $^{\text{T}}$ (95.1%). According to the well-accepted bacterial species delimitation of less than 97% 16S rRNA gene sequence similarity (Wayne *et al.*, 1987), strains WG^{T} and Ycb08 could represent a novel species affiliated either to the genus *Selenomonas* or *Mitsuokella*.

Based on the genus descriptions, members of *Mitsuokella* are characterized by a cell morphology of stout rods without flagella (Mitsuoka *et al.*, 1974; Holdeman *et al.*, 1984; Lan *et al.*, 2002), while cells of the genus *Selenomonas* are curved to helical rods, motile with a typical flagellar arrangement as a tuft near the centre of the concave side (Bryant, 1984; Kingsley & Hoeniger, 1973). Strain WG^{T} presented crescent-shaped cells with flagella on the concave side; together with other differential characteristics listed in Table 1, the two strains can be classified in the genus *Selenomonas*. Furthermore, the two strains resembled other members of the genus *Selenomonas* in metabolic features, such as the use of a variety of carbohydrates as substrates and the production of acetate, propionate and CO_2 from glucose, as well as production of H_2S from L-cysteine but the absence of nitrate reduction (Table 1). However, strains WG^{T} and Ycb08 displayed <97% 16S rRNA gene sequence similarity with described *Selenomonas* species and differed from their closest relative, *S. ruminantium* subsp. *ruminantium* DSM 2150 $^{\text{T}}$, in their higher G+C content and by liquefying gelatin but not fermenting dulcitol, mannitol, sorbitol and xylose. Thus, on the basis of DNA G+C content and the physiological and phylogenetic traits described above, strains WG^{T} and Ycb08 represent a novel

Fig. 1. Phylogenetic tree of *Selenomonas* species and related organisms based on a 1379 bp fragment of the 16S rRNA gene, showing the position of strains WG^T and Ycb08. The tree was rooted with *Bacillus subtilis* DSM 10^T and constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) were also recovered by the maximum-parsimony and minimum-evolution methods. Numbers at nodes represent percentages of bootstrap support based on 1000 resampled datasets of the neighbour-joining method. GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence.

species of the genus *Selenomonas*, for which the name *Selenomonas bovis* sp. nov. is proposed.

Description of *Selenomonas bovis* sp. nov.

Selenomonas bovis (bo'vis. L. gen. n. *bovis* of a cow, of a bovine, referring to the isolation of the first strains from yak rumen contents).

Gram-negative, crescent-shaped rods, 4–6 µm long and 0.9–1.1 µm wide. No spore formation is observed. Flagella (four to six) are arranged linearly as a tuft near the centre

of the concave side of the cell. The temperature range for growth is 27–46 °C; optimum growth at 39 °C. Grows at pH 4.2–8.3, with optimum growth at pH 7.0–7.2. The generation time is 2.3 h when grown on glucose at 39 °C. The final pH in PYG medium is 4.5. Utilizes arabinose, glucose, mannose, cellobiose, lactose, sucrose, trehalose, melibiose, raffinose, salicin and aesculin but not lactate, xylose, starch, dulcitol, mannitol or sorbitol. The end products of glucose fermentation are acetate, propionate and CO₂ according to the equation 1 glucose → 0.7 acetate + 1.1 propionate + 1.2 CO₂. Liquefies gelatin and

Table 1. Differential characteristics of strains WG^T and Ycb08 and phylogenetically related type strains

Strains: 1, strain WG^T; 2, strain Ycb08; 3, *S. ruminantium* subsp. *ruminantium* DSM 2150^T (data from Bryant, 1956, 1984); 4, *S. ruminantium* subsp. *lactilytica* DSM 2872^T (unless indicated, data from Bryant, 1956, 1984); 5, *S. sputigena* DSM 20758^T (unless indicated, data from Bryant, 1956, 1984); 6, *M. jalaludinii* DSM 13811^T (Lan *et al.*, 2002); 7, *M. multacida* NCTC 10934^T (Mitsuoka *et al.*, 1974; Holdeman *et al.*, 1984).

Characteristic	1	2	3	4	5	6	7
Isolation source	Yak	Yak	Cattle	Cattle	Human	Cattle	Human
Morphology*	CR	CR	CR	CR	CR	SR	SR
Flagellar arrangement†	LA	LA	LA	LA	LA	NF	NF
Voges–Proskauer test	–	–	–	–	–‡	+	+
Nitrate reduction	–	–	–	–	+	+	+
H ₂ S production	+	+	+	+	–	–	–
Gelatin liquefaction	+	+	–	–	–	–	–
Acid production from:							
Glycerol	–	–	–	+	+	+	–
Dulcitol	–	–	+	+	–	–	–
Mannitol	–	–	+	+	+	–	+
Sorbitol	–	–	+	+	–	+	–
Xylose	–	–	+	+	–	+	+
Mannose	+	+	+	+	–	+	+
Raffinose	+	–	+	+	+	–	+
Inulin	+	–	+	+	+	–	–
DNA G + C content (mol%)	63.9 ± 0.2	62.5 ± 0.2	54.2 ± 0.2	55.2 ± 0.4	61	56.8	57.3

*CR, Curved rods; SR, stout rods.

†LA, Linearly arranged as a tuft near the centre of the concave side; NF, no flagella.

‡Data from this study.

produces H₂S but not indole. Does not reduce nitrate. Voges–Proskauer and methyl red tests are negative. The G+C content of the genomic DNA of the type strain is 63.9±0.2 mol%.

The type strain, WG^T (=CGMCC 1.5073^T =JCM 15470^T), and a second strain, Ycb08, were isolated from yak rumen contents.

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