Mitsuokella jalaludinii sp. nov., from the rumens of cattle in Malaysia

G. Q. Lan, Y. W. Ho and N. Abdullah

Five strains of phytase-producing, Gram-negative, non-spore-forming, non-motile, small, stout, rod-shaped, strictly anaerobic, fermentative bacteria were isolated from the rumens of cattle in Malaysia. All five strains had morphological, physiological and biochemical features in common. Although these strains had many physiological and biochemical characteristics that were identical to those of the *Mitsuokella multacida* type strain (ATCC 27723T), they could be distinguished from this species by means of the following characteristics: a smaller cell size (12–24 µm long and 0.6–0.8 µm wide); a lower final pH value (3.8–4.0) in peptone/yeast extract/glucose broth; inhibition by 0.001% brilliant green; insensitivity to kanamycin (100 µg ml⁻¹) and penicillin (10 µg ml⁻¹); a higher optimum growth temperature (approx. 42 °C); the ability to grow at 45 and 47 °C; the ability to ferment glycerol, sorbitol and amido; and the inability to ferment mannitol, rhamnose, D-tagatose and melezitose. The G+C content of the type strain (M 9⁷) of these five strains was 56.9 mol%. Analysis of the 16S rRNA gene sequence of type strain M 9⁷ indicated that the strain falls within the genus *Mitsuokella*. The sequence similarity between type strain M 9⁷ and *Mitsuokella multacida* was 98.7%. The DNA–DNA relatedness between type strain M 9⁷ and *Mitsuokella multacida* type strain DSM 20544T (= ATCC 27723) was 63.8%, indicating that, in spite of a high level of similarity for the 16S rRNA gene sequence, type strain M 9⁷ is independent of *Mitsuokella multacida* at the species level. On the basis of these results, a new species, *Mitsuokella jalaludinii* sp. nov., is proposed for these strains. The type strain is M 9⁷ (= DSM 13811T = ATCC BAA-307).}

Keywords: *Mitsuokella jalaludinii*, rumen bacteria, phytase-producing bacteria

Mitsuoka *et al.* (1974) isolated a large number of bacterial strains from the faeces of humans, pigs, dogs and chickens; these strains appeared to be identical to, or closely related to, *Megamonas hypermegas* (basonym: *Bacteroides hypermegas*). These Gram-negative, non-spore-forming, non-motile, strictly anaerobic, stout rods could ferment a wide range of carbohydrates and were named *Bacteroides multiacidus* by Mitsuoka *et al.* (1974). Later, Shah & Collins (1982) assigned *B. multiacidus* to a new genus, *Mitsuokella*, and named it *Mitsuokella multiacidus*, which was recently corrected to *Mitsuokella multacida* by Euzéby (1998). In the process of screening for phytase-producing bacteria from the rumens of cattle in Malaysia, five isolates were found to be related to *Mitsuokella multacida*. To verify the systematic position of these phytase-producing bacterial isolates, a study of their morphological and physiological characteristics, 16S rRNA gene sequence, G+C contents and DNA–DNA hybridization was undertaken.

For the isolation of phytase-producing rumen bacteria, rumen fluid was sampled from fistulated cattle (*Bos indicus*) fed 60% commercial concentrate and 40% oil palm frond for 15 d. The samples were taken 4 h after the onset of morning feeding. The anaerobic techniques described by Hungate (1950, 1969) for rumen bacteria, with the modifications of Bryant & Burkey (1953) and Bryant (1972), were used for isolation and maintenance of the bacterial cultures. The rumen fluid was serially diluted to 10⁻⁹; 0.1 ml was inoculated into roll-tubes containing modified phytase-screening
Acid production from carbohydrates was determined most of the physiological studies was PYG medium. The medium used for preparation of inocula and for 10°C anaerobic conditions and incubated at 39°C by Sieo et al. (1999). The bacterial cells were harvested and prepared for scanning electron microscopy using the methods described by Siew et al. (1999). The bacterial strains for 48 h. The volatile and non-volatile fatty acids produced from the fermentation of PYG broth containing 1% glucose were determined by GC (GC-14A; Shimadzu) using the method described by Kageyama et al. (1973). Other physiological characteristics studied included acetylmethylcarbinol production (Voges–Proskauer test), nitrate reduction, catalase production, indole production, H₂S production, urease production, Tween 80 hydrolysis, gelatin liquefaction, arginine decarboxylase production, growth in 4.5% NaCl, and growth at 20, 45 and 47 °C. The media used for the determination of these physiological characteristics are shown in Table 1. Growth stimulation by bile or glucose, determined by adding 2% bile or 1% glucose to peptone/yeast (PY) medium (Holdeman et al., 1977), was also studied. The growth was monitored by measuring the optical density (OD₆₆₀) using a spectrophotometer (DU-65; Beckman). The stimulation effects were determined by comparing the optical density (OD₆₆₀) values of the experimental and control tubes. Tolerance to inhibitory substances was determined in PYG broth containing one of the following substances: 0.001% brilliant green, 0.005% crystal violet, neomycin (25 µg ml⁻¹), kanamycin (100 µg ml⁻¹), penicillin (10 µg ml⁻¹), erythromycin (50 µg ml⁻¹) and bacitracin (3 µl ml⁻¹). All the tests in the physiological studies were repeated three times, each with duplicates.

The nucleotide sequence of the 16S rRNA gene of one strain i.e. type strain M 9T was determined. The methods used for genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and

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<td>Urease production</td>
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* All media were bubbled with oxygen-free CO₂ gas before autoclaving, and the pH was adjusted before use if necessary.

(MPS) agar medium. The MPS medium was modified from the phytase-screening medium of Howson & Davis (1983) by the addition of 1 g peptone (Difco) and 37.5 ml vitamin-free casein 1-1 medium. The inoculated tubes were incubated at 39 °C for 6 d. After the incubation period, bacterial colonies with surrounding clear zones were transferred to modified M10 (MM 10) medium and incubated at 39 °C for 24 h. The MM 10 medium was modified from Medium 10 (Caldwell & Bryant, 1966) by replacing K₂HPO₄ and KH₂PO₄ with sodium phytate. Pure cultures were obtained by repeated reinoculation and reisolation in roll-tubes containing MPS agar medium. Pure cultures were stored at −70 °C in MM 10 medium and subcultures were made every 4 months. The type strain of Mitsuokella multacida, ATCC 27723 T (=NCTC 10934 T = DSM 20544 T) obtained from the American Type Culture Collection, was used as the reference strain in the morphological and physiological studies.

For the morphological study, pure bacterial strains were streaked on peptone/yeast extract/glucose (PYG) agar medium (Holdeman et al., 1977) under anaerobic conditions and incubated at 39 °C in an atmosphere of 100% CO₂ for 2 d. The cellular appearance was determined from Gram-stained smears examined with a light microscope (Leitz Aristoplan). Colonies were examined for size, colour and elevation. Morphological characteristics and size were further studied with a scanning electron microscope (JSM 6400; JEOL). The bacterial strains were grown on PYG agar medium at 39 °C for 48 h, after which the bacterial cells were harvested and prepared for scanning electron microscopy using the methods described by Sieo et al. (1999).

The medium used for preparation of inocula and for most of the physiological studies was PYG medium. Acid production from carbohydrates was determined by using the API kit system (bioMérieux). The formation of gas in the fermentation of glucose (1%) was detected in PYG broth by the appearance of bubbles in a Durham tube. The final pH values were measured electrometrically in PYG broth by incubating the bacterial strains for 48 h. The volatile and non-volatile fatty acids produced from the fermentation of PYG broth containing 1% glucose were determined by GC (GC-14A; Shimadzu) using the method described by Kageyama et al. (1973). Other physiological characteristics studied included acetylmethylcarbinol production (Voges–Proskauer test), nitrate reduction, catalase production, indole production, H₂S production, urease production, Tween 80 hydrolysis, gelatin liquefaction, arginine decarboxylase production, growth in 4.5% NaCl, and growth at 20, 45 and 47 °C.

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purification of the PCR products were similar to those described previously by Rainey et al. (1996). Purified PCR products were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) as directed by the manufacturer's protocol. Sequence reaction products were electrophoresed using the Applied Biosystems 373A DNA Sequencer. The resulting sequence data were manually aligned using the Alignment Editor ae2 (Maidak et al., 1996) and compared with representative 16S rRNA gene sequences of organisms belonging to the Gram-positive bacteria (Maidak et al., 1996). For comparison, 16S rRNA gene sequences were obtained from the EMBL database or the database of the Ribosomal Database Project (Maidak et al., 1996). The 16S rRNA gene-similarity values were calculated by pairwise comparison of the sequences within the alignment. For construction of the phylogenetic dendrogram, the Phylipeny Inference Package (PHYLIP) (Felsenstein, 1993) was used. Pairwise evolutionary distances were computed from percentage similarities by the correction of Jukes & Cantor (1969), and, on the basis of the evolutionary distance values, the phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The root of the tree was determined by including the 16S rRNA gene sequence of Bacillus subtilis in the analysis.

For studies on the G+C content (mol %) of the DNA of type strain M 9T, cells were disrupted by passage through a French pressure cell (Aminco) and the DNA was then purified on hydroxyapatite according to the procedures of Cashion et al. (1977) and Visuvanathan et al. (1989). The DNA was hydrolysed with P, nuclease and the nucleotides were dephosphorylized with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides were analysed by using HPLC apparatus fitted with an LKB 2150 high-pressure pump and an LKB 2151 UV detector (270 nm) connected to a Shimadzu CR-3A integrator. The Nucleosil 100-5C18 analytical column (250 x 4 mm) was equipped with a Nucleosil 100-5C18 pre-column (20 x 4 mm; MELZ VDS). The chromatography running temperature was 26 °C, the solvent used was 0.6 M NH4H2PO4/acetonitril (80:6, v/v; pH 4.4), the flow rate was 0.7 ml min⁻¹, and the pressure was approximately 140 bars (Tamaoka & Komagata, 1984). A non-methylated Lambda-DNA with a G+C content of 49.858 mol % (Sigma) was used for calibration. The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) according to the method of Mesbah et al. (1989).

DNA–DNA hybridization was also determined. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Huss et al. (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermostyrometer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992).

From a total of 125 bacterial strains isolated from the rumens of cattle, five strains which showed strong abilities to hydrolyse sodium phytate were studied and identified. Observations using light microscopy and scanning electron microscopy showed that all five strains had features in common. They were Gram-negative, non-spore-forming, non-motile, small, short rods (1–2 x 2–4 µm long and 0.6–0.8 µm wide) with rounded ends and occurred singly, in pairs, in short chains or in irregular groups (Fig. 1). Colonies on PYG agar were circular with regular margins, greyish-white, convex with smooth surfaces and 1–4 mm in diameter after 48 h incubation at 39 °C.

Abundant growth occurred under strict anaerobic conditions. Although some growth occurred under aerobic conditions, it was not sustained after about four or five subcultures. The optimum temperature for growth was 42 °C and growth was still good at 47 °C. Growth was markedly enhanced by the presence of glucose in the medium, but not by bile.

**Fig. 1.** Scanning electron micrographs showing the rod forms of (top) Mitsuokella multacida ATCC 27723T and (bottom) Mitsuokella jalaludinii sp. nov. Bar, 10 µm.
The other physiological and biochemical characteristics of the strains are summarized in Table 2. Many of the characteristics were similar to those of the Mitsuokella multacida type strain (ATCC 27723T), particularly the fermentation products of glucose. However, there were a number of characteristics that were distinctly different and could be used to distinguish these strains from the Mitsuokella multacida type strain (ATCC 27723T), as follows: a smaller cell size (1.2–2.4 μm in length and 0.6–0.8 μm in width); a lower final pH value (3.8–4.0) in PYG broth; inhibition of growth by 0.001% brilliant green; insensitivity to kanamycin (100 μg ml\(^{-1}\)) and penicillin (10 μg ml\(^{-1}\)); a higher optimum growth temperature (approx. 42 °C); the ability to grow at 45 and 47 °C; the ability to ferment glycerol, sorbitol and amidon; and the inability to ferment mannitol, rhamnose, D-tagatose and melezitose (Table 2).

Approximately 95% of the 16S rRNA gene sequence of type strain M 9\(^{T}\) was determined and was found to comprise 1543 nucleotide bases. Comparison of the 16S rRNA gene sequence of type strain M 9\(^{T}\) with those of some related taxa demonstrated that the strain was closely related to Mitsuokella multacida, having a sequence similarity of 98.7%. The phylogenetic dendrogram based on the evolutionary distance value shows that strain M 9\(^{T}\) belongs to the Clostridium sub-phyllum of the Gram-positive bacteria (Fig. 2). Within this sub-phyllum, strain M 9\(^{T}\) is a member of the Sporomusa sub-branch with the highest 16S rRNA gene sequence similarity with Mitsuokella multacida. Thus, the phylogenetic dendrogram clearly shows that type strain M 9\(^{T}\) falls within the genus Mitsuokella and that the sequence similarity between M 9\(^{T}\) and Mitsuokella multacida is higher than 97%, suggesting that they are closely related and that DNA–DNA hybridization studies should be carried out to verify the taxonomic relationship between these two strains.

The G+C content of the DNA of strain M 9\(^{T}\) was determined as 56.9 mol%, which was similar to that of...
Mitsuokella multacida type strain A 405-1T (= ATCC 27723T) (57·3 mol%; Mitsuoka et al., 1974).

The genomic DNA–DNA relatedness between strain M 9T and Mitsuokella multacida type strain DSM 20544T (= ATCC 27723T) was determined. The DNA–DNA hybridization value between strain M 9T and Mitsuokella multacida type strain DSM 20544T (= ATCC 27723T) was 63·8%. This low hybridization value indicates that strain M 9T is independent of Mitsuokella multacida at species level.

On the basis of the results of this study, it can be seen that a number of biochemical and physiological characteristics, as well as the smaller size and the DNA–DNA hybridization value, clearly distinguish strain M 9T from Mitsuokella multacida, which is the sole species in the genus. Therefore, it is evident that strain M 9T is a previously undescribed Mitsuokella species, for which we propose the name Mitsuokella jalaludinii.

**Description of Mitsuokella jalaludinii sp. nov.**

*Mitsuokella jalaludinii* (jal.al.u.di’ni.i. N.L. gen. n. jalaludinii of Jalaludin, in honour of S. Jalaludin, an animal nutritionist and Vice-Chancellor of Universiti Putra Malaysia, who has contributed significantly to rumen microbiology).

Gram-negative, non-spor-forming, non-motile, small, stout rods (1·2–2·4 μm in length and 0·6–0·8 μm in width). Cells occur singly, in pairs, in short chains or in irregular groups. Colonies on PYG agar after 2 d incubation are greyish-white, roughly circular and convex with smooth surfaces and regular edges. Strictly anaerobic. The final pH in PYG broth is 3·8–4·0. There is no copious gas formation from glucose. The major end-products from glucose fermentation are lactic acid, succinic acid and some acetic acid. Growth is stimulated by fermentable carbohydrates but not by bile. The optimum growth temperature is 42°C. Growth occurs at 45 and 47°C. Gelatin liquefaction, indole production, catalase production, urease production, hydrogen sulfide production, Tween 80 hydrolysis, arginine acid decarboxylase and growth in 4·5% sodium chloride are negative. The nitrate-reduction test, the methyl red test and the Voges–Proskauer reaction are positive. Glycerol, L-arabinose, ribose, D-xylene, galactose, glucose, fructose, D-mannose, inositol, sorbitol, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, D-raffinose, amidon, D-turanose, D-arabitol and 5-keto-glucuronate are fermented. Erythritol, D-arabinose, L-xylene, adonitol, methyl β-xyloside, L-sorbose, rhamnose, dulcitol, mannitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylgalactosamine, amygdalin, inulin, melezitose, glycinogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, gluconate and 2-keto-glucuronate are not fermented. Resistant to kanamycin, neomycin, penicillin, bacitracin and erythromycin. Intolerant to 0·005% crystal violet and 0·001% brilliant green. Full 16S rRNA gene sequence similarity and DNA–DNA relatedness to Mitsuokella multacida are 98·7 and 63·8%, respectively. The G+C content of the DNA is 56·8 mol%.

Isolated from the rumens of cattle. The type strain is M 9T (= DSM 13811T = ATCC BAA-307T).

**References**


