Megamonas rupellensis sp. nov., an anaerobe isolated from the caecum of a duck

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We report here the identification, characterization and culture of a Gram-negative to Gram-variable, rod-shaped, non-spore-forming anaerobic bacterium (strain FM1025^T) isolated from the caecum of a duck. Phylogenetic analysis based on comparative 16S rRNA gene sequencing showed that this strain clustered with species of the family 'Acidaminococcaceae', with 94.9% similarity to *Megamonas hypermegale* DSM 1672^T and less than 91% similarity with type strains of *Pectinatus* species. Sequence similarities of at least 98–99% were observed with numerous sequences deposited in GenBank of uncultured strains from human and chicken caecal contents, but this strain is the first isolate of this taxon to be cultivated and described. On the basis of morphological, physiological and phylogenetic features, this strain should be assigned to a novel species in the genus *Megamonas*, for which the name *Megamonas rupellensis* sp. nov. is proposed. The type strain is strain FM1025^T (=DSM 19944^T =CIP 109788^T).

The microbial composition of the chicken intestine evolves with age and varies in its different sections. It is estimated that 60–90% of the intestinal bacterial community can not currently be cultivated (Lu *et al.*, 2003). The dominant micro-organisms in the gut include bacteria related to *Clostridium*, *Lactobacillus*, *Bacteroides* and *Megamonas* (Lan *et al.*, 2002; Zhu *et al.*, 2002). This complex microflora is known to play a key role in the health of birds. For instance, young chickens are very sensitive to enteropathogen infections because their intestinal micro-organisms are not fully established (Nurmi & Rantala, 1973). In 2000, searching for bacteria that produce anti-enteropathogen molecules, Portrait *et al.* (2000) isolated an anaerobic strain (FM1025^T) from the caecum of a duck. This strain was preliminarily identified as *Fusobacterium mortiferum* using API galleries. In the present work, we have characterized this bacterium further and, using 16S rRNA gene sequence analysis, we have shown that strain FM1025^T represents a novel species belonging to the genus *Megamonas* (Shah & Collins, 1982).

Strain FM1025^T was grown routinely at 37 °C on TGY broth (w/v; 3% tryptone, 2% yeast extract, 0.5% glucose, 0.05% L-cysteine hydrochloride) using either anaerobic jars (AnaeroGen; Oxoid) or an anaerobic chamber containing 10% H₂, 10% CO₂ and 80% N₂ (MACS DG500; Don Whitley Scientific). Strain FM1025^T was

restreaked weekly onto TGY agar [TGY broth solidified with 1.2% (w/v) agar] and could be stored for several years after mixing 0.5 ml of an overnight culture with 0.5 ml 30% (w/v) glycerol. Unless otherwise specified, growth of strain FM1025^T was tested in 250 ml TGY broth incubated at 37 °C under continuous stirring (160 r.p.m.) in the anaerobic chamber.

Strain FM1025^T grew optimally at 37 °C and the pH range for growth was pH 5–9 with an optimum at pH 7. On solid M63 minimal medium (Miller, 1972) supplemented with the reducing amino acid L-cysteine hydrochloride (0.05% w/v), no colonies were observed after 72 h of incubation. In the same medium enriched with 0.2% yeast extract, colonies could be visualized after 48 h of incubation. Strain FM1025^T was anaerobic, non-motile and oxidase- and catalase-negative.

After growth on solid medium or in liquid broth for 12, 24 or 48 h, cultures were Gram-negative to Gram-variable. The strain was unsporulated in TGY but intracellular inclusions could be seen on Gram stain preparations. For electron microscopy, 5 ml aliquots of a 48 h culture were centrifuged for 5 min at 5000 g. Cell pellets were washed twice with 50 mM sodium cacodylate buffer (pH 7) and prepared as described by Dongard & Bordes (1991). The preparations were examined using a Philips Quanta 200 ESEM/FEG microscope with an Everhart Thornley-BSE detector. Cells were rod-shaped (1.0–6.0 µm) and occurred singly, in pairs and in filaments in liquid cultures (Fig. 1). Longer filaments were observed in solid cultures.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FM1025^T is EU346729.

Fig. 1. Scanning electron micrographs of cells of strain FM1025^T grown anaerobically for 48 h in TGY broth. The arrow indicates a filamentous structure. Bars, 20 µm (top) and 2 µm (bottom).

DNA was prepared as described by Marmur (1961) and the G+C content of the DNA was determined by HPLC (Carlotti *et al.*, 1993). The 16S rRNA gene was amplified by PCR using primers Fpna1 (5'-AAGTCGAACGGGGT-GTTTA-3') and RPna2 (5'-CAAACCCGAAGCCGGCTG-3'). Amplified DNA fragments were purified using the Qiagen PCR purification kit and both strands were sequenced on an ABI 310 sequencer (Applied Biosystems) using the 16S rRNA full gene sequencing kit from ABI. For identification of closest relatives, the sequence of strain FM1025^T was compared to 16S rRNA gene sequences in GenBank using BLASTN version 2.2.17 (Altschul *et al.*, 1997). Sequence alignment was performed using CLUSTAL W. Phylogenetic trees were constructed using PAUP 4.0, on 1399 base comparisons using the neighbour-joining method and bootstrap values of 1000 replicates. The sequence of the 16S rRNA gene determined for strain FM1025^T (1399 nt) placed it near the genus *Megamonas* within the phylum *Firmicutes*, in the lineage *Clostridia*, *Clostridiales*, *Acidaminococcaceae* (Morotomi *et al.*, 2007).

The closest relatives were *Megamonas hypermegale* DSM 1672^T, at 94.9% similarity and 71 nucleotide differences, and the recently described *Megamonas funiformis* YIT 11815^T (Sakon *et al.*, 2008), at 97.6% similarity and 29 nucleotide differences. The 100% bootstrap value observed strongly supports the grouping of FM1025^T with *Megamonas hypermegale*, the type species of the genus (Cato & Barnes, 1976; Harrison & Hansen, 1963; Shah & Collins, 1982). Sequence similarity of less than 91% was observed with representative type strains of *Pectinatus* and *Selenomonas* species (Fig. 2).

This difference in 16S rRNA gene sequence suggests a phylogenetic difference sufficient to assign strain FM1025^T to a novel species in the genus *Megamonas*. This is supported by the DNA G+C content of 33 ± 2 mol% (Shah & Collins, 1982). However strain FM1025^T (1.0–6.0 µm) can be easily differentiated from *M. hypermegale* ($2\text{--}4.6 \times 11.6\text{--}15$ µm) by its smaller cells (Cato & Barnes, 1976; Harrison & Hansen, 1963) and on the basis of an absence of acid production from melezitose and L-rhamnose. It can be differentiated from *M. funiformis* (Sakon *et al.*, 2008) on the basis of the presence of acid production from glycerol and cellobiose. 16S rRNA gene sequence comparisons revealed similarity above 98% with numerous sequences of uncultured isolates from the caeca of broiler chickens (Bjerrum *et al.*, 2006) or other types of organisms. Strain FM1025^T is the first isolate of this taxon to be cultivated and described.

API 20A profiles were analysed after 24 and 48 h and submitted to bioMérieux for identification. The profiles obtained were similar to those of the Gram-negative *Prevotella ruminicola* subsp. *ruminicola* and the Gram-positive *Actinomyces israelii* (80.2% ID in both cases). Sensitivity towards antibiotics was tested using disc assays. A 24 h culture in TGY broth was diluted to an OD₆₀₀ of 0.12 as specified by the recommendations of the Comité de l'Antibiogramme of the Société Française de Microbiologie. TGY agar plates were flooded with 2 ml of this dilution prior to placing the antibiotic discs (Bio-Rad). Inhibition haloes were measured after 24 h incubation in the anaerobic chamber. Strain FM1025^T was resistant to vancomycin at 30 µg, similar to some *Prevotella*, *Bacteroides* and *Fusobacterium* species and *Clostridium innocuum*. Strain FM1025^T was sensitive to 25 µg amoxicillin, 10 µg imipenem, 4 µg metronidazole, 30 µg chloramphenicol, 20 µg amoxicillin and 10 µg clavulanic acid.

Static TGY broth cultures (18 ml) were used for fermentation analysis. Anaerobic jars and long glass tubes were preferred to the anaerobic chamber and Erlenmeyer flasks in order to minimize the loss of volatile by-products. Aliquots of cultures (48 h, 37 °C) were centrifuged for 5 min at 7500 g. Culture supernatants were sterile-filtered (0.22 µm) and analysed in triplicate using a gas chromatograph (Hewlett Packard 5890A) as described by Erable *et al.* (2005). Glucose concentration was evaluated by the DNS

Fig. 2. Phylogenetic tree showing the position of strain FM1025^T, other species of the 'Acidaminococcaceae' and *Megamonas hypermegale* DSM 1672^T, based on 16S rRNA gene sequence similarities. The tree was constructed by using the neighbour-joining method, with bootstrap values (reliability values of internal branches) expressed as percentages of 1000 replicates. Accession numbers are shown in parentheses. Bar, 1 % sequence divergence. *Anaerobacter polyendosporus* DSM 5272^T was used as the outgroup.

(dinitrosalicylic acid) method (Miller, 1959). We were unable to detect the presence of lactate, propanol, acetone, butyrate, 2,3-butanediol or pentanoate in the supernatant of strain FM1025^T. Hence, FM1025^T can also be differentiated from *M. funiformis* (Sakon *et al.*, 2008) and *M. hypermegale* (Harrison & Hansen, 1963) by the absence of lactate as an end product from TYG broth. Acetate and propionate were the only organic acids detected from glucose fermentation. Propionate appeared to be the major fermentation end product, with a concentration of 25 mM after 48 h compared with 20 mM for acetate. The initial concentration of glucose was 28 mM and reached 1.3 mM after 72 h of culture. Considering that 90 % of the glucose consumed (26.3 mM) was converted into end products (the rest being converted into biomass), we assessed that 95 % was fermented into acetate and propionate. This confirmed that acetate and propionate were the major organic acids produced by strain FM1025^T in TGY medium. Moreover, the final pH of cultures was 4.6–4.9, corresponding to the pK_a of acetate ($pK_a=4.75$) and propionate ($pK_a=4.87$).

Description of *Megamonas rupellensis* sp. nov.

Megamonas rupellensis (ru.pel.len'sis. M.L. fem. adj. *rupellensis* referring to La Rochelle, the site of isolation of the type strain).

Obligately anaerobic, mesophilic, saccharophilic, non-spore-forming, non-motile, Gram-negative or Gram-variable, long rods (1.0–6.0 μ m). Cells occur singly, in pairs or in chains in liquid medium. Growth occurs in TGY broth within 24 h. On solid medium, colonies are 'ecru' and notched, 10–20 mm in diameter after 2 days of growth at

37 °C. End products are acetate and propionate. The pH for growth is pH 5.0–9.0, with an optimum at pH 7.0. Oxidase and catalase activities are absent. Nitrate and nitrite reduction activities are absent. Acids are produced from D-glucose, D-mannitol, D-lactose, sucrose, maltose, salicin, D-xylose, L-arabinose, glycerol, cellobiose, D-mannose, raffinose, D-sorbitol and trehalose, but not from melezitose or L-rhamnose. Resistant to vancomycin (30 μ g per disc), but not to amoxicillin (25 μ g), imipenem (10 μ g), metronidazole (4 μ g), chloramphenicol (30 μ g), amoxicillin (20 μ g) or clavulanic acid (10 μ g). The G+C content of DNA of the type strain is 33 ± 2 mol%.

The type strain, FM1025^T (=DSM 19944^T =CIP 109788^T), was isolated from the caecum of a duck in La Rochelle, France.

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