Veillonella magna sp. nov., isolated from the jejunal mucosa of a healthy pig, and emended description of Veillonella ratti

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A bacterium, designated strain lac18T, was isolated in pure culture from the mucosal jejunum of a healthy pig, using a medium selective for anaerobic lactic acid bacteria and containing porcine gastric mucin as the main carbon and nitrogen source. Cells of this strain were coccus-shaped, arranged singly or in pairs and were Gram-stain-negative, oxidase-negative, non-spore-forming, anaerobic and microaerotolerant. An analysis based on 16S rRNA gene sequences indicated that strain lac18T should be assigned to the genus Veillonella, class Clostridia, phylum Firmicutes. 16S rRNA and dnaK gene sequence-based phylogenetic analyses both indicated that the most closely related species were Veillonella ratti ATCC 17746T (similarities of 96.6 and 84.5 %, respectively) and Veillonella criceti ATCC 17747T (similarities of 96.6 and 83.4 %, respectively). The results of DNA–DNA hybridizations between strain lac18T and these Veillonella species and the type species of the genus, Veillonella parvula DSM 2008T, confirmed the genotypic distinctness of the novel isolate. Data from phenotypic studies also served to differentiate strain lac18T from related isolates. Therefore strain lac18T represents a novel species of the genus Veillonella, for which the name Veillonella magna sp. nov. is proposed. The type strain is lac18T (=CCUG 55454T=CIP 109767T=DSM 19857T=JCM 15053T).

The anaerobic, Gram-staining-negative cocci of the genus Veillonella Prévot (Prévot, 1953) belong phylogenetically to the Sporomusa sub-branch of the class Clostridia of the Gram-type-positive bacteria (Stackebrandt et al., 1985; Willems & Collins, 1995). The taxonomy of the genus is based on extensive genotypic DNA–DNA hybridization studies performed by Mays et al. (1982) and reaffirmed recently by Byun et al. (2007). At the time of writing, the genus comprises the type species Veillonella parvula (Veillon & Zuber, 1898; Prévot, 1933; Mays et al., 1982) and ten other species: Veillonella alcalescens [Lewkowicz, 1901; Prévot, 1933; subsequently reclassified as a later heterotypic synonym of V. parvula (Mays et al., 1982)], V. atypica, V. criceti, V. dispar, V. ratti, V. rodentium (Rogosa, 1965; Mays et al., 1982), V. caviae (Mays et al., 1982), V. montpellieriensis (Jumas-Bilak et al., 2004), V. denticariosi (Byun et al., 2007) and V. rogosae (Arif et al., 2008).

The type strains of all recognized Veillonella species have been isolated from their main natural habitats, namely the oral cavity and the gastrointestinal tract of homeothermic vertebrates (Smith, 1965; Rogosa, 1984; Kolenbrander, 2006). The species V. parvula, V. atypica, V. dispar, V. montpellieriensis, V. denticariosi and V. rogosae have been isolated from man and V. criceti, V. ratti, V. rodentium and V. caviae have been isolated from rodents. In food animals, Veillonella strains are also detected regularly as indigenous inhabitants of all sections of the gastrointestinal tract (Jonhs, 1951; Alexander & Davies, 1963; Smith & Jones, 1963; Tannock & Smith, 1970; McGillivery & Cranwell, 1992; Leser et al., 2002; Tsukahara & Ushida, 2002; Murphy et al., 2005; Gong et al., 2007; Wise & Siragusa, 2007).

The veillonellae possess a ‘truncated glycolytic system’ (Rogosa et al., 1965) but utilize the metabolic end products of co-existing carbohydrate-fermenting bacteria, i.e. three- and four-carbon organic acids, as energy and carbon sources via the methylmalonyl-CoA pathway (Delwiche et al., 1985; Denger & Schink, 1992; Seeliger et al., 2002). In the gastrointestinal tract, strains of the genus Veillonella thereby constitute an essential link with indigenous lactic acid bacteria in a natural microbial food-chain (Marounek & Bartos, 1987; Pacheco Delahaye et al., 1994; Kolenbrander, 2006). Mixed cultures of veillonellae and, inter alia, lactic acid bacteria, isolated from the caeca of

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Definitive identification of strain lac18T, including its human or non-rodent origin.

Species of the genus Veillonella was further resolved by means of partial sequence (703 bp) analysis of the 70 kDa heat-shock-protein gene (dnaK) as proposed by Marchandin et al. (2003). Phylogenetic analysis in BLAST was carried out as described for the 16S rRNA gene. As there is currently no published threshold for dnaK gene sequence-based species delineation for the genus Veillonella (Marchandin et al., 2005), a pairwise BLAST 2 SEQUENCES analysis of all known dnaK sequences in the genus was also performed (http://www.ncbi.nlm.nih.gov/; Tatusova & Madden, 1999). DNA–DNA hybridization with V. ratti DSM 20736T, V. criceti DSM 20734T and V. parvula DSM 2008T was determined using a membrane filter technique (Johnson, 1994) with digoxigenin-based products from Roche (DIG DNA labelling kit, DIG Easy Hyb granules, Anti-DIG-AP, CDP-Star) according to the manufacturer’s protocols. The amount of genomic DNA immobilized on the nylon membranes was 4000 ng. The actual hybridization was performed at 42 °C for 12 h. Signal intensities were visualized by means of long exposure to a charge-coupled device camera (SensiCam Qe, CamWare software version 2.20; PCO) and the similarities (%) were determined using the PCRBAS software program (version 2.09e; Raytest).

The 16S rRNA gene sequence analysis indicated that strain lac18T was most closely related to the V. ratti–V. criceti cluster (Johnson & Harich, 1983) of the genus Veillonella, with V. ratti ATCC 17746T (96.6 %), V. criceti ATCC 17747T (96.6 %), V. denticariosi RBV106T (95.1 %) and V. atypica ATCC 17744T (95.1 %) as the closest relatives with validly published names. The sequence similarity with respect to V. parvula ATCC 10790T was 94.0 %. High similarity (99.4 %) was obtained with the 1309 bp sequence of one unpublished isolate, Veillonella sp. MY-P9 (GenBank accession no. DQ979378 deposited by M. Y. Jung & Y.-H. Chang, 2006). The genetic distinctness of strain lac18T with respect to the V. ratti–V. criceti cluster was strongly supported by a high bootstrap value (100 %) in the neighbour-joining analysis (Fig. 1). The dnaK gene sequence analysis indicated that the taxonomic position of the novel strain was closer to V. ratti ATCC 17746T (84.5 %) than to V. criceti ATCC 17747T (83.4 %). The sequence similarity of the novel strain was highest with the Veillonella sp. isolate MY-P9 (99.7 %). The BLAST 2 SEQUENCES analysis showed that the interspecific similarity of the dnaK gene sequences of recognized strains in the genus Veillonella (except Veillonella sp. ADV 4313.2, Veillonella sp. ADV 360.1 and Veillonella sp. MY-P9) varied between 77.9 % (lac18T versus V. dispar ATCC 17748T) and 95.7 % (V. denticariosi RBV81 versus V. rodentium ATCC 17743T). The intraspecific similarities of chickens, are inhibitory to enteropathogenic bacteria (Hinton et al., 1991; Hinton et al., 1992; Corrier et al., 1995). One such mixed culture is commercially available as a health-promoting competitive exclusion culture for poultry production (Nisbet, 2002).

Strain lac18T was isolated in 2007 in the course of a cultural study of lactic acid bacteria from porcine mucosal jejenum. A cross-bred fattening pig was reared at our Institute and euthanized at 62 days by means of an overdose of sodium pentobarbital, in accordance with the legal requirements of the relevant local authority for animal welfare. Fresh, washed, pea-size mucosal samples were inoculated into 10 ml of a medium selective for lactic acid bacteria and containing porcine gastric mucin (Type III; Sigma) as the main carbon and nitrogen source. This medium, henceforth referred to as mucin medium, contained the following (g−1): 10.0 g mucin, 0.01 g peptone, 0.01 g yeast extract, 0.01 g glucose, 0.3 g NaCl, 0.1 g CaCl2, 6.0 g KH2PO4, 5 ml Rogosa’s salt solution (Rogosa et al., 1951), 1 ml modified (lacking elements already included in Rogosa’s salt solution) Pfennig’s SL8 trace element solution (Bast, 2001), 0.2 ml vitamin solution (Stams et al., 1993) and 0.5 × 10−3 g resazurin. All of the components were autoclaved, except the vitamins, which were filter-sterilized. The pH of the medium was adjusted to 5.0 using 37–38 % HCl prior to autoclaving. Incubations were performed in loosely screw-capped 15 ml tubes at 37 °C under anaerobic, CO2-enriched (18 % by vol.) conditions in an anaerobic jar using Anaerocult A gas packs (Merck). The incubation time between initial transfers was always 7–14 days. Single colonies were obtained on mucin medium containing 0.4–0.75 % agar. Strain lac18T was eventually recovered from a co-culture with an Enterococcus isolate (lac32) on MRS agar (Roth). It was presumptively identified to the genus level using the VITEK Anaerobe Identification system (bioMérieux). Strain lac18T appeared to be distinct from a total of 32 isolates that were recovered from the gastric and jejunal mucosae of two pigs (data not shown). We decided, therefore, to classify and characterize strain lac18T by means of a polyphasic approach. On the basis of the data from this study, we describe the first species of the genus Veillonella with a type strain of non-human or non-rodent origin.

Definitive identification of strain lac18T, including its taxonomic assignment to the genus Veillonella, was obtained using 16S rRNA gene sequence analysis. Genomic DNA was extracted from cells with a NucleoSpin Tissue kit (Macherey-Nagel). The 16S rRNA gene was amplified using a HotStarTaq Master Mix kit (Qiagen) and primers 27f and 1492r (Lane, 1991). Amplified PCR products were purified using a High Pure PCR Product Purification kit (Roche) and sequenced commercially (MWG Biotech). A 16S rRNA gene sequence of 1449 bp was obtained and subsequently analysed with the Ribosomal Database Project (RDP) II classification algorithm (http://rdp.cme.msu.edu/; Maidak et al., 2001; Wang et al., 2007) and the BLAST algorithm (http://www.ncbi.nlm.nih.gov/; Altschul et al., 1990; McGinnis & Madden, 2004). Phylogenetic analysis with MEGA software (version 4.0) (Tamura et al., 2007) was conducted to infer an evolutionary tree using the neighbour-joining method (Saitou & Nei, 1987) based on the Kimura two-parameter model (Kimura, 1980). The taxonomic position of strain lac18T in the genus Veillonella was further resolved by means of partial sequence (703 bp) analysis of the 70 kDa heat-shock-protein gene (dnaK) as proposed by Marchandin et al. (2003). Phylogenetic analysis in BLAST was carried out as described for the 16S rRNA gene. As there is currently no published threshold for dnaK gene sequence-based species delineation for the genus Veillonella (Marchandin et al., 2005), a pairwise BLAST 2 SEQUENCES analysis of all known dnaK sequences in the genus was also performed (http://www.ncbi.nlm.nih.gov/; Tatusova & Madden, 1999). DNA–DNA hybridization with V. ratti DSM 20736T, V. criceti DSM 20734T and V. parvula DSM 2008T was determined using a membrane filter technique (Johnson, 1994) with digoxigenin-based products from Roche (DIG DNA labelling kit, DIG Easy Hyb granules, Anti-DIG-AP, CDP-Star) according to the manufacturer’s protocols. The amount of genomic DNA immobilized on the nylon membranes was 4000 ng. The actual hybridization was performed at 42 °C for 12 h. Signal intensities were visualized by means of long exposure to a charge-coupled device camera (SensiCam Qe, CamWare software version 2.20; PCO) and the similarities (%) were determined using the PCRBAS software program (version 2.09e; Raytest).
Veillonella magna sp. nov. from pig jejunum

Fig. 1. Neighbour-joining phylogenetic tree, based on partial 16S rRNA gene sequences, showing the relationships between strain lac18T (1449 bp), all type strains of the genus Veillonella and isolate Veillonella sp. MY-P9. Acidaminococcus fermentans ATCC 25085T (1488 bp) was used as the outgroup organism. Bootstrap percentages (based on 1000 replicates) are shown at nodes. Bar, genetic distance of 2 %.

the dnaK gene sequences of strains of recognized species varied between 98.4 % and 99.8 % (V. montpellierensis). This comparison produced a 96–98 % threshold for dnaK gene-based species delineation in the genus Veillonella (see Supplementary Table S1, available in IJSEM Online). DNA–DNA hybridization results confirmed the genotypic distinctness of strain lac18T within the genus Veillonella: the DNA–DNA relatedness values with V. ratti DSM 20736T, V. criceti DSM 20734T and V. parvula DSM 2008T were 40.0, 39.0 and 27.0 %, respectively.

Phenotypic studies on strain lac18T were conducted mainly in comparison with V. ratti DSM 20736T and V. criceti DSM 20734T (which were purchased, for this purpose, from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Unless stated otherwise, the standard cultivation medium used was a modified version of Veillonella medium (Rogosa, 1956) (i.e. without antibiotic and basic fuchsin and supplemented with 0.001 g resazurin l−1 and 0.003 g putrescine l−1).

Cell morphology was observed under a Zeiss light microscope at ×1000 magnification and under a digital scanning electron microscope (DSM 950; Zeiss) at ×5000–20 000 magnification (Taras, 2001). Gram-staining was performed using a Gram-colour staining set (Merck) according to the manufacturer’s instructions. Spore formation and motility were analysed using conventional methods (Bast, 2001). Commercial tests (Fluka) were applied for the detection of cytochrome oxidase and nitrate reduction. The benzidine reaction with porphyrin and catalase activity were assayed according to Deibel & Evans (1960). The production of H2O2 was studied as described by Juárez Tomás et al. (2004) with the following exceptions: prolonged incubation was performed under both anaerobic and microaerobic (8–10 % CO2, 5–7 % O2, by vol.; Anaerocult C gas packs, Merck) conditions for up to 4 days and exposure to air was for 24 h. The H2O2 assays were evaluated as described by Otero & Nadermacías (2006). H2S production was analysed in a defined modified Veillonella medium with 0.6 × 10−3 M l-cysteine (Rogosa & Bishop, 1964a). For biochemical profiling, the Rapid ID 32A and API ZYM systems (bioMérieux) were used according to the manufacturer’s instructions. Blood agar base No. 2 (LabM) plus 5 % defibrinated horse blood was chosen as the second culture medium with Rapid ID 32A. Putrescine auxotrophy was analysed in the defined modified Veillonella medium, supplemented with putrescine at 0.003 and 0.005 g l−1 (Rogosa & Bishop, 1964b; Ritchey & Delwiche, 1975). Fermentation of fructose was studied likewise using supplementation with 0.02 M D-fructose. Short-chain fatty acids produced as metabolic end products from the fermentation of lactate were determined as described previously (Schäfer, 1995) and gas production was assayed by measuring increases in pressure in air-tight, screw-capped roll tubes. Growth characteristics under various pH and temperature values were studied using standard anaerobic methods (Hungate, 1969). The response to oxygen was assessed using aerobic and microaerobic (Anaerocult C gas packs) incubation with or without the reducing agent sodium thioglycolate (0.75 g l−1).

The results of the phenotypic characterization, including differential features, are given in the species description. Phenotypic characteristics that serve to differentiate strain lac18T from V. ratti DSM 20736T and V. criceti DSM 20734T are given in Table 1. On the basis of the results of 16S rRNA and dnaK gene sequencing and the differential phenotypic data, strain lac18T represents a novel species of...
the genus *Veillonella*, for which the name *Veillonella magna* is proposed.

**Description of Veillonella magna** sp. nov.

*Veillonella magna* (mag’na. L. fem. adj. magna big, referring to the cell size in comparison with other species of the genus *Veillonella*).

Cells are spherical to coccoid, 0.65–0.85 μm in diameter (mean, approx. 0.75 μm) and are arranged singly or in pairs (rarely in short chains or small masses). Adjacent sides of cell pairs are flattened and the cell surface is convoluted (Fig. 2). Cells stain Gram-negative, with an enhanced tendency to resist decolorization after 48 h incubation. They are non-spore-forming and non-motile.

After anaerobic incubation on modified *Veillonella* agar at 37 °C for 24 h, colonies are circular, up to 1.2 mm in diameter, have entire margins and smooth, shiny surfaces and are raised, opaque, creamy grey, soft and moist. After 48 h, colonies look similar but are up to 2.0 mm in diameter and greyish beige. Cells are non-haemolytic on blood agar. Cytochrome oxidase is not present. Nitrate is reduced. The benzidine test for porphyrin is positive. Catalase activity is negative or delayed (approx. 30–45 s) and weak. The type of catalase is not determined in the

**Table 1. Results of differential phenotypic characterization of strain lac18T, V. ratti DSM 20736T and V. criceti DSM 20734T**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Cell diameter (μm)</td>
<td>0.65–0.85</td>
<td>0.3–0.5*</td>
<td>0.3–0.5*</td>
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<tr>
<td>Catalase</td>
<td>− or delayed and w</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Accumulation of H₂O₂</td>
<td></td>
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<tr>
<td>Anaerobic</td>
<td>w</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Microaerobic</td>
<td>Moderate w</td>
<td>w</td>
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<tr>
<td><strong>Biochemical profile (API ZYM)</strong></td>
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<tr>
<td>Alkaline phosphatase</td>
<td>3.4 ± 0.7</td>
<td>2.3 ± 1.0</td>
<td>2.5 ± 0.5</td>
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<tr>
<td>Esterase (C4)</td>
<td>1.1 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>2.2 ± 0.4</td>
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<tr>
<td>Esterase lipase (C8)</td>
<td>0.1 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>Leucine arylamidase</td>
<td>1.4 ± 0.8</td>
<td>1.3 ± 0.9</td>
<td>0.6 ± 0.5</td>
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<tr>
<td>Valine arylamidase</td>
<td>0.1 ± 0.3</td>
<td>0.2 ± 0.4</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>4.1 ± 0.5</td>
<td>4.1 ± 0.9</td>
<td>3.8 ± 0.4</td>
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<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>3.3 ± 0.5</td>
<td>4.7 ± 0.5</td>
<td>3.4 ± 0.7</td>
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<tr>
<td>Putrescine requirement</td>
<td>+</td>
<td>−*</td>
<td>+</td>
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<tr>
<td>Fermentation of fructose</td>
<td></td>
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<tr>
<td>Change of pH at 28 days†</td>
<td>−0.01 ± 0.01</td>
<td>−0.29 ± 0.09</td>
<td>−0.32 ± 0.02</td>
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<tr>
<td>Temperature range for growth (on modified <em>Veillonella</em> medium)</td>
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<tr>
<td>45 °C (agar)</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>40 °C (broth), OD max††</td>
<td>1.38 ± 0.04</td>
<td>0.87 ± 0.02</td>
<td>0.88 ± 0.02</td>
</tr>
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</table>

*Data taken from Holt et al. (1994); cell diameter as generally given for the genus *Veillonella*.†Values are means ± SD, n=3.‡OD was measured at 600 nm with an Ultrospec 2000 spectrophotometer (Pharmacia Biotech).
benzidine test. Benzidine reacts non-specifically with the porphyrin compounds of the haem group in respiratory systems including (besides catalase) cytochromes and nitrite reductase (Bascomb & Manafi, 1998). H$_2$O$_2$ accumulation is weak under anaerobic conditions and moderate under microaerobic conditions. H$_2$S is produced from l-cysteine. Biochemical profiling with Rapid ID 32A yields positive results for arginine dihydrolase, reduction of nitrates, alkaline phosphatase and histidine arylamidase (identification profile 2000500001). The leucine arylamidase reaction is ambiguous using modified Veillonella medium [identification profile 200050(0/2)001]. API ZYM results are indicated in Table 1. Putrescine is required for normal growth. Negative for fermentation of fructose. Lactate is metabolized anaerobically to more propionate and less acetate. Gas is also produced, often abundantly. The temperature range for growth is approximately 21–45 °C. Growth is very weak at approximately 21 °C, absent at 46 °C, good at 30 °C and very good at 37 and 40 °C. The pH range for growth is 5.5–9.5 (optimum, pH 6.5–7.5). Alkalization of modified Veillonella medium occurs at pH 5.5–6.0 (maximally to pH 6.5) and acidification occurs at pH 6.5–9.5 (maximally to pH 6.2). Cells are anaerobic and microaerotolerant (less than approx. 5% O$_2$, by vol.), showing no growth on agar plates but producing vigorous growth and long-lasting viability (up to at least 2 weeks) in broth medium suspended in air-tight, screw-capped roll tubes under aerobic, unreduced conditions at 37 °C.

The type strain, lac$^{18T}$ (=CCUG 55454$^{T}$=CIP 109767$^{T}$=DSM 19857$^{T}$=JCM 15053$^{T}$), was isolated from the mucosal jejenum of a healthy pig in Berlin, Germany.

Emended description of *Veillonella ratti* (Rogosa 1965) Mays et al. 1982

The description remains as given by Rogosa (1984), except that the strain *V. ratti* DSM 20736$^{T}$ is capable of fructose fermentation.

Acknowledgements

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