Veillonella seminalis sp. nov., a novel anaerobic Gram-stain-negative coccus from human clinical samples, and emended description of the genus Veillonella

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Ten isolates of unknown, Gram-stain-negative, anaerobic cocci were recovered from human clinical samples, mainly from semen. On the basis of their phenotypic features, including morphology, main metabolic end products, gas production, nitrate reduction and decarboxylation of succinate, the strains were identified as members of the genus Veillonella. Multi-locus sequence analysis and corresponding phylogenies were based on 16S rRNA, dnaK and rpoB genes, and on the newly proposed gltA gene. The strains shared high levels of genetic sequence similarity and were related most closely to Veillonella ratti. The strains could not be differentiated from V. ratti on the basis of 16S rRNA gene sequence analysis while gltA, rpoB and dnaK gene sequences showed 85.1, 93.5 and 90.2% similarity with those of the type strain of V. ratti, respectively. Phylogenetic analyses revealed that the isolates formed a robust clade in the V. ratti–Veillonella criceti–Veillonella magna subgroup of the genus Veillonella. As observed for V. criceti, the isolates were able to ferment fructose. In contrast to other members of the genus Veillonella, the 10 strains were not able to metabolize lactate. Cellular fatty acid composition was consistent with that of other species of the genus Veillonella. From these data, the 10 isolates are considered to belong to a novel species in the genus Veillonella, for which the name Veillonella seminalis sp. nov. is proposed. The type strain is ADV 4313.2T (=CIP 107810T =LMG 28162T). Veillonella strain ACS-216-V-Col6b subjected to whole genome sequencing as part as the Human Microbiome Project is another representative of V. seminalis sp. nov. An emended description of the genus Veillonella is also proposed.

The genus Veillonella belongs to the family Veillonellaceae in the phylum Firmicutes (Marchandin et al., 2010; Marchandin & Jumas-Bilak, 2014). Species of the genus Veillonella are members of the oral, genito-urinary, respiratory and/or intestinal microbiota of humans and other mammals. At the time of writing, 12 species have been described in the genus Veillonella. Species were recov ered either from human samples (Veillonella denticiariosi, Veillonella dispar, Veillonella montpellierensis, Veillonella rogosae and Veillonella tohetsusensis) or from non-human animal samples (Veillonella caviae, Veillonella criceti, Veillonella magna, Veillonella ratti and Veillonella rodentium), Veillonella atypica and Veillonella parvula being

Abbreviations: CFA, cellular fatty acid; DMA, dimethyl acetyl; ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA, dnaK, rpoB and gltA gene sequences of Veillonella seminalis sp. nov. ADV 4313.2T are AY211542, AY793381, GU479185 and KJ580452, respectively.

One supplementary table and two supplementary figures are available with the online version of this paper.
isolated from both human and other animal origin (Rogosa, 1984; Jumas-Bilak et al., 2004; Byun et al., 2007; Arif et al., 2008; Kraatz & Taras, 2008; Mashima et al., 2013). The genus groups anaerobic Gram-stain-negative cocci characterized by their ability to reduce nitrate in contrast to other genera including Gram-negative-staining cocci, i.e. Acidaminococcus, Anaeroglobus, Megasphaera and Negativicoccus. However, species of the genus Veillonella cannot be differentiated from each other by routine phenotypic means because of the lack of discriminative features. In addition, some closely related species, such as V. denticiariosi and V. rodentium, V. ratti and V. criceti, and V. dispar and V. parvula, cannot be differentiated on the basis of 16S rRNA gene sequencing because they share at least 99 % of their rrs gene nucleotide bases (Marchandin et al., 2005; Byun et al., 2007; Michon et al., 2010). Finally, intra-chromosomal heterogeneity between the four 16S rRNA gene copies found in the genus Veillonella and/or intraspecific rrs gene variability that may surpass interspecific variability has been demonstrated, thereby impairing the 16S rRNA-based identification of closely related species (Marchandin et al., 2003; Michon et al., 2010). Therefore, molecular-based identification methods based on housekeeping genes such as dnaK and rpoB were developed and supported the description of novel species as well as increased knowledge on the relative distribution or habitat of species of the genus. For example, in both cultivation-dependent and cultivation-independent studies of the oral cavity based on rpoB gene sequencing, the predominant species were V. atypica, V. dispar and V. rogosae while V. parvula, previously thought to be a common inhabitant of the Veillon microbiota, was very rarely identified (Beighton et al., 2008; Mashima et al., 2011).

Here, we performed a polyphasic taxonomic study of 10 isolates of anaerobic, Gram-negative, nitrate-reducing cocci isolated from human clinical samples, which represent a novel species in the genus Veillonella. Among them, isolate ADV 4313.2T was previously described as the first human isolate belonging to the V. ratti–V. criceti group, and it had been suggested that it may represent a novel taxon within the genus Veillonella on the basis of low levels of dnaK gene sequence similarity with its closest relatives (Marchandin et al., 2005).

Routine phenotypic analysis results
Isolates were recovered from diverse human clinical samples, mainly in semen (n=7) and among polymicrobial cultures, in ten patients (one female and nine male; age range 1.5 months to 50 years) hospitalized at the University Hospital of Montpellier, France, between August 2002 and May 2013. The three isolates not recovered from semen were from a Bartholin gland abscess, a perianal abscess and a groin wound.

The isolates were cultured on Columbia sheep blood agar (bioMérieux) at 37 °C in an anaerobic jar with the AnaeroGen System (Oxoid), which reduces the oxygen level to below 0.1 % within 2.5 h and results in 7–15 % carbon dioxide. After 2 days of incubation, circular, opaque, greyish, shiny, non-haemolytic and smooth colonies 1 mm in diameter with entire margins and a central elevation were observed. Gram staining showed Gram-stain-negative, coccoid (0.5–0.8 μm in diameter) to ovoid-shaped cells of 0.8 × 0.9–1.2 μm mainly arranged as single cells, pairs or short chains. Spores were not observed and cells were non-motile. None of the isolates displayed catalase activity. Nitrate reduction was observed for all isolates using Diatabs (Rosco Diagnostica). Five isolates including strain ADV 4313.2T were able to grow under microaerobic conditions tested using a Campygen Compact system (final atmosphere reduced to 6 % oxygen, and supplemented with 14 % carbon dioxide) (Oxoid). Susceptibility to special-potency discs determined as described by Jousimies-Somer et al. (2002) showed all the isolates to be susceptible to 1 mg kanamycin and 4 μg metronidazole discs but resistant to 5 μg vancomycin, 10 μg colistin and 1 mg sodium polyanethol sulfonate discs. Susceptibility to the 1 mg bile disc was variable (three resistant isolates including strain ADV 4313.2T out of the 10 studied). Using Microflex matrix-assisted laser desorption ionization-time of flight MS (Bruker Daltonics) with MALDI Biotyper for identification, V. ratti was the first and only proposed species for the isolates; however, no score >2.3 indicating satisfactory confidence for species identification was obtained (score range 1.65–2.08).

Because resistance to the colistin disc is rarely observed in members of the genus Veillonella and V. ratti was not previously identified from human clinical samples, the isolates were subjected to further polyphasic investigations.

Genetic analyses
DNA extraction was performed using a MasterPure DNA purification kit (Epicentre Biotechnologies) as recommended by the supplier. 16S rRNA, 70 kDa heat-shock protein (dnaK) and RNA polymerase B (rpoB) genes were amplified by PCR as previously described (Carlier et al., 2002; Marchandin et al., 2003; Michon et al., 2010). For the gltA gene encoding cytochrome synthase, primers were designed in this work from alignment of the eight Veillonella strains with available whole genome sequences at the time of primer design listed hereafter, using CLUSTAL W in the BioEdit program, version 7.1.9 (Hall, 1999) (http://www.mbio.ncsu.edu/bioedit/bioedit.html): Veillonella sp. 3_1_44 (GenBank accession number NZ_ADCV00000000), Veillonella sp. 6_1_27 (NZ_ADCH00000000), Veillonella sp. oral taxon 158 str. F0412 (NZ_AENU00000000), V. parvula DSM 2008T (NC_013520), V. parvula ACS-068-Sch12 (NZ_AEXI00000000), V. parvula ATCC 17745T (NZ_ADFU00000000) and V. dispar ATCC 17748T (NZ_ACIK00000000). Primers gltA 3406F (5’-GGGTGTAAAATGGCTCGTGT-3’) and gltA 4271R (5’-GCATAGCCACCGGACATAC-3’) were designed using Primer3 software (http://bioinfo.ut.ee/primer3/)
The species to the group formed by the clinical isolates. BioEdit revealed that similarity tables constructed using utilities implemented in by the same two bases from those of the eight other strains. The 16S rRNA gene sequence similarity between strain ADV 4313.2T and the two of the 10 isolates had 3528 International Journal of Systematic and Evolutionary Microbiology rrns were compared for type strains of all other species of the genus Veillonella including V. ratti–V. criceti–V. magna and the 10 clinical isolates. The 16S rRNA gene-based ML tree, gltA-based ML tree and NJ tree reconstructed from concatenated 16S rRNA, dnaK, rpoB and gltA gene sequences are shown in Figs S1 and S2 (available in the online Supplementary Material) and Fig. 1, respectively. Within this clade, the clinical isolates formed a tight group supported by high bootstrap values of 99 or 100 % depending on the analysis and by a phylogenetic branch clearly separated from that of V. ratti except in the 16S rRNA gene-based phylogeny. The overall topology of the trees based on housekeeping genes suggested the phylogenetic grouping of the 10 clinical isolates within the same species. Therefore, these 10 strains are hereafter referred to as belonging to a novel species in the genus Veillonella, for which we propose the name Veillonella seminalis sp. nov.

**Complementary phenotypic investigations**

Biochemical reactions were performed according to the procedures of the VPI Anaerobe Laboratory Manual (Holdeman et al., 1977) by using trypticase-yeast extract-haemin (TYH) medium supplemented with 1 % (w/v) of each sterilized substrate. Oxidase detection was performed using oxidase discs purchased from Bio-Rad. The Rapid ID 32A kit (API bioMérieux) was used for enzyme profile determination as recommended by the manufacturer. Metabolic end products were assayed by quantitative GC as described by Carlier (1985). Analysis of the cellular fatty acid (CFA) composition was performed at the BCCM/LMG public collection. Cells were grown for 48 h at 35 °C on supplemented brain heart infusion with blood (BHIBLA) plates, under anaerobic conditions. Inoculation and harvesting of the cells, and extraction and analysis were performed according to the recommendations of the commercial identification system MIDI, except that cells were harvested from the whole plate to obtain a sufficient concentration of fatty acids in the extract. The whole-cell fatty acid composition was determined by GC. The peak-naming table MIDI BHIBLA 3.80 was used.

Urease activity and indole production were not detected. Oxidase activity was not detected. Gelatin was not liquefied and milk was not modified. Desulfuroviridin was not produced. Aesculin was not hydrolysed. Fructose was the only

**Phylogenetic analyses**

The 16S rRNA gene (1306 bp), the dnaK gene (541 bp), the rpoB gene (600 bp), the gltA gene (698 bp) and the concatenation of these four housekeeping gene (3145 bp) sequences of the clinical strains were compared with those of the type strains of species of the genus Veillonella. Evolutionary distances were analysed using the neighbour-joining (NJ) (Kimura two-parameter substitution model) and maximum-likelihood (ML) (general time-reversible substitution model plus gamma distribution and invariant sites) using phylogenetic analyses available at http://www. phylogeny.fr (Dereeper et al. 2008). Bootstrap support was computed after 100 or 1000 reiterations for ML and NJ analysis, respectively. Dialister succinatophilus YIT 11850T (GenBank accession number ADLT01000000) was used as the outgroup micro-organism in all phylogenetic analyses.

Phylogenetic trees based on individual or concatenated gene sequences (16S rRNA, dnaK, gltA and rpoB) reconstructed by ML or distance methods were congruent, revealing two clades in the genus Veillonella, one of them including V. ratti–V. criceti–V. magna and the 10 clinical isolates. The 16S rRNA gene-based ML tree, gltA-based ML tree and NJ tree reconstructed from concatenated 16S rRNA, dnaK, rpoB and gltA gene sequences suggested the phylogenetic grouping of the 10 clinical isolates within the same species.
Veillonella seminalis sp. nov.

Quantitative GC allowed the detection of acetic acid (14–45.1 mmol l\(^{-1}\)), propionic acid (30–113.1 mmol l\(^{-1}\)) and trace amounts of 2-hydroxyvaleric acid (0.9–1.3 mmol l\(^{-1}\)) as metabolic end products from TYH with glucose (TGYH). Lactate was not fermented, only trace amounts of propionate (0.3–0.44 mmol l\(^{-1}\)) being formed from lactate. Succinate was fermented to propionate (87.9–98.8 mmol l\(^{-1}\)) and 2-hydroxyvaleric acid (4.4–5.4 mmol l\(^{-1}\)).

The CFA composition was determined for four isolates including ADV 4313.2\(^{T}\) that showed similar results (Table S1). Compared with available data published (Carlier, 2009), major discrepancies concerning C\(_{14:0}\) dimethyl acetyl (DMA) and C\(_{12:0}\)10:8 were revealed. We compared these results with CFA patterns available from the website of the CCUG collection for type strains of eight species of the genus Veillonella grown on chocolate agar at 37 \(^\circ\mathrm{C}\) and showed that C\(_{14:0}\) DMA is present in all the strains analysed, ranging from 4.9 to 13.2 % of the strain total CFA content (http://www.ccug.se/). Our results were also reanalysed using the peak naming table MIDI TSBA50 (v5.0) and, depending on the database used, the major CFA found in the four strains corresponded either to summed feature 8 (26.78–35.53 % of total CFAs) or to C\(_{17:1}\)08c (29.12–38.04 %), a result congruent with CCUG results showing C\(_{17:1}\)08c to be a major CFA, representing up to 43.6 % of the total CFAs of V. criceti CCUG 56973\(^{T}\) (data not shown). Our results were therefore considered consistent with those previously reported for other species of the genus Veillonella, with the major CFA being summed feature 8 or C\(_{17:1}\)08 depending on the database used, and C\(_{13:0}\) (Carlier, 2009).

The proposed novel species displayed rarely encountered or atypical features in the genus Veillonella. Resistance to a 10 \(\mu\)g colistin disc was observed for V. ratti and V. montpellierensis only. Absence of lactate fermentation was not previously reported for any members of the genus Veillonella. In the clade formed by V. seminalis–V. ratti–V. criceti–V. magna, V. seminalis sp. nov. is the only species including human isolates. Indeed, V. ratti and V. criceti are exclusively reported from the mouth and intestine of rodents, and V. magna has been characterized from the jejunal mucosa of a healthy pig (Kraatz & Taras, 2008). The novel species appears to be related to the human genital tract, and more distantly to the digestive tract, as
previously noted for *V. montpeliereensis*, supporting the proposal of *V. seminalis* as the name for the novel species characterized here. The phenotypic characteristics differentiating *V. seminalis* sp. nov. from the closest genetically and phylogenetically related species *V. ratti*, *V. criceti* and *V. magna* are detailed in Table 1.

**Complete genome sequence analysis of a strain phylogenetically related to *V. seminalis***

The whole genome of *V. ratti* strain ACS-216-V-Col6b has been sequenced as a reference genome for the Human Microbiome Project (GenBank accession number NZ_AHAF01000000). The strain was isolated from a vaginal swab and grown on Schaedler agar at 37°C under anaerobic conditions. Available data showed that this strain has identical 16S rRNA gene sequences to the 10 clinical isolates described herein while its *rpoB* gene sequence differed by two bases. All phylogenetic analyses showed the phylogenetic grouping of strain ACS-216-V-Col6b in *V. seminalis* sp. nov. and thus supported the strain to be another representative of the proposed novel species.

Strain ACS-216-V-Col6b has a DNA G+C content of 41.8 mol% (http://www.ncbi.nlm.nih.gov/genome/14507?project_id=182889), which is a relatively high value for the genus *Veillonella*. Indeed, DNA G+C values between 36 and 40 mol% were observed for most species in the genus *Veillonella* except for two species displaying higher values: *V. rodentium* (42–43 mol%) and *V. ratti* (41–43 mol%) (Carlier, 2009).

**Emended description of the genus *Veillonella***

Prévot 1933 emend. Mays *et al.* 1982

The description is as described by Prévot (1933) and emended by Mays *et al.* (1982) except that lactate may or may not be fermented and that growth under microaerobic conditions may occur.

**Description of *Veillonella seminalis* sp. nov.**

*Veillonella seminalis* (se.mi.na’lis. L. fem. adj. seminalis pertaining to semen, the main source of isolation for the species).

Cells are non-motile, non-spore-forming and coccoid (0.5–0.8 μm in diameter) to ovoid-shaped (0.8 × 0.9–1.2 μm). Cells are mainly arranged in pairs or short chains. Colonies on Columbia blood agar are circular, opaque, greyish, non-haemolytic, shiny and smooth, 1 mm in diameter with entire margins and a central elevation after 48 h of incubation at 37°C in an anaerobic atmosphere. Cells are anaerobic and may be facultatively microaerophilic. Catalase and oxidase activities are not detected. Reduction of nitrates is

**Table 1. Characteristics differentiating *V. seminalis* sp. nov. from related species**

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
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<tbody>
<tr>
<td><strong>Cell morphology</strong></td>
<td>Cocci (0.5–0.8 μm in diameter) to ovoid (0.8 × 0.9–1.2 μm)</td>
<td>Cocci (0.3–0.5 μm in diameter)*</td>
<td>Cocci (0.3–0.5 μm in diameter)*</td>
<td>Spherical to coccoid (0.65–0.85 μm in diameter)†</td>
</tr>
<tr>
<td><strong>Growth under microaerobic conditions</strong></td>
<td>v (±5/10)‡</td>
<td>+</td>
<td>+</td>
<td>+ or delayed and weak†</td>
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<tr>
<td>Catalase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td><strong>Susceptibility to special-potency discs</strong></td>
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<td>Colistin (10 μg)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
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<tr>
<td>Bile (1 mg)</td>
<td>v‡</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<tr>
<td>Lactate fermentation</td>
<td>–</td>
<td>+*</td>
<td>+*</td>
<td>+ †</td>
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<tr>
<td>Ability to ferment fructose</td>
<td>–</td>
<td>–*</td>
<td>+*</td>
<td>– †</td>
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<td><strong>Rapid ID 32 A results</strong></td>
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<td>PAL</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+ †</td>
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<td>ADH</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ †</td>
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<tr>
<td>HisA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ †</td>
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<tr>
<td><strong>Major cellular fatty acids</strong></td>
<td>Summed feature 8 or C₁₇:09, C₁₃:0 DMA</td>
<td>C₁₇:09, C₁₃:0, C₁₅:0*</td>
<td>C₁₇:09, C₁₃:0, C₁₅:0*</td>
<td>ND</td>
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<td>(Table S1)</td>
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*Data from Carlier (2009).

†Data from Kraatz & Taras (2008).

‡*Veillonella seminalis* sp. nov. ADV 4313.2T grew under microaerobic conditions and displayed bile resistance.
positive, alkaline phosphatase is present and gas is produced. Resistant to 10 μg colistin discs. Fructose is the only carbohydrate fermented. Lactate is not fermented. Decarboxylation of succinate is observed. Major metabolic end products from TGYH broth are acetate and propionate. Major CFAs are summed feature 8 containing one or more of C_{17:1}ω9c and/or C_{17:1}ω8 (MIDI HBIHLA 3.80 peak naming table) or C_{17:1}ω8 (MIDI TBSA50 database), C_{13:0} and C_{14:0} DMA. Can be differentiated from other species of the genus *Veillonella* by the absence of lactate fermentation, and on the basis of dnaK, rpoB and gltA gene sequencing. Isolated from human clinical samples mainly originating from the genital tract, particularly from semen.

The type strain is ADV4313.2^T (=CIP 107810^T=LMG 28162^T). The DNA G+C content of strain ACS-216-V-Colb6 is 41.8 mol%.

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**References**


