Ruminococcus gauvreauii sp. nov., a glycopeptide-resistant species isolated from a human faecal specimen

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A novel strictly anaerobic, vancomycin-resistant, Gram-positive coccus (strain CCRI-16110T) was isolated from a human faecal specimen. This strain was characterized using morphological, biochemical and molecular taxonomic methods. The organism was unable to hydrolyse aesculin and failed to produce acid from cellobiose, D-lactose and α-rhamnose. Acetic acid was the sole product of glucose fermentation by the organism. On the basis of 16S rRNA and tuf gene sequence comparison, strain CCRI-16110T was most closely related to species of the genus Ruminococcus and formed a hitherto unknown sublineage within the Clostridium cocoides rRNA cluster of organisms (cluster XIVa). Based on phenotypic and phylogenetic evidence, a novel species, Ruminococcus gauvreauii sp. nov., is proposed. The type strain is CCRI-16110T (=NML 060141T = CCUG 54292T = JCM 14987T).

The majority of species described so far in the human gut microbiota consists of obligate anaerobes that can be assigned to the Bacteroides group, the Clostridium cocoides group (cluster XIVa) or the Clostridium leptum group (cluster IV) (Collins et al., 1994; Eckburg et al., 2005; Franks et al., 1998; Hold et al., 2002; Maukonen et al., 2006; Suau et al., 1999). Cluster XIVa represents one of the most important clostridial clusters and contains a combination of species of intermixed genera including Clostridium, Ruminococcus, Eubacterium, Acetitomaculum, Roseburia and Coprococcus (Collins et al., 1994).

During a surveillance programme at the Montreal General Hospital (Quebec, Canada) in 2001 to detect glycopeptide-resistant enterococci carriers using a PCR primer pair specific for the vancomycin-resistance vanD gene, a high prevalence of vanD-positive faecal specimens containing no vancomycin-resistant enterococci was found (Domingo et al., 2005b). One of the vanD-positive faecal specimens (rectal swab ERV-110) obtained from a patient of this hospital was further processed. No further information was obtained from this patient. The initial processing and isolation procedure of specimen ERV-110 has been reported previously (Domingo et al., 2005b). Briefly, a 300 μl aliquot of a rectal swab suspension was cultured in brain-heart infusion (BHI) broth supplemented with 1 μg vitamin K ml⁻¹ and 5 μg haemin ml⁻¹ (eBHII) and incubated at 35 °C in an anaerobic atmosphere for 24 h. The eBHII culture was subcultured onto eBHII agar (eBHIA) containing 32 μg vancomycin ml⁻¹ and incubated anaerobically for 3 days. A colony that was positive for vanD by PCR was recovered and appeared impure, as shown by the presence of different cell morphologies upon Gram staining (Domingo et al., 2005b). This vanD-positive microbial cell consortium was repeatedly subcultured on eBHIA, but the vanD-positive strains could not be isolated in pure culture from this cell consortium at that time (Domingo et al., 2005b). To favour the growth of vanD-positive strains against other members of the consortium, a new enriched medium containing BHI broth supplemented with 1 μg vitamin K ml⁻¹, 5 μg haemin ml⁻¹, 0.5 mg

Abbreviations: DMA, dimethyl acetal; FF, formate–fumarate; MIC, minimal inhibitory concentration.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and tuf gene sequences of strain CCRI-16110T are EF529620 and EF529615, respectively.

Phylogenetic trees based on the 16S rRNA gene sequences constructed using the neighbour-joining and maximum-parsimony algorithms are available as supplementary material with the online version of this paper.
Cells of strain CCRI-16110T were organized as single cocci (MIDI) as described previously (Bernard et al., 2005b). The eleventh subculture in eBHILPVA broth incubated for 4 days was plated onto eBHILPVA agar. This plate led to the isolation of a single colony after incubation under anaerobic conditions for 7 days. This colony was shown to be positive for vanD by PCR. This isolate was picked and streaked out twice to ascertain purity. A pure isolate (strain CCRI-16110T) was further processed for identification.

Phenotypic identification tests of strain CCRI-16110T were performed as described in the Wadsworth and VPI anaerobic manuals (Holdeman et al., 1977; Jousimies-Somer et al., 2002) at the Centre de Recherche en Infectiologie de l’Université Laval (Québec, Canada) and at the National Microbiology Laboratory (Winnipeg, Canada). Morphology was observed using an optical Leitz light microscope and transmission electron microscopy (JEOL 1200EX). Degree of growth was observed in peptone-yeast extract (PY) broth enriched with glucose (PYD) with or without bile, serum, Tween 80 or formate-fumarate (FF). Special-potency discs for colistin (10 μg), kanamycin (1000 μg), vancomycin (5 μg), sodium polyanethol sulfonate (5 %) and metronidazole (5 μg), all from Oxoid, were used as recommended in the Wadsworth manual (Jousimies-Somer et al., 2002). Minimal inhibitory concentrations (MICs) of vancomycin and metronidazole were determined by the agar dilution method for anaerobes according to the Clinical and Laboratory Standards Institute (NCCLS, 2004), whereas the MIC for teicoplanin was determined by the Etest method (AB Biodisk). Fermentation tests of different sugars were performed using pre-reduced, anaerobically sterilized PY sugar broth tubes from Med-Ox Diagnostics. The isolate was examined with pre-formed enzyme tests including the Rapid ID 32A and API ZYM systems (bioMérieux), as well as the RapID ANA II from Remel (Oxoid), according to the manufacturer’s instructions. GLC analysis of metabolic end products of fermentation was done as described previously (Bernard et al., 2002), except that anaerobe system broth was acquired from Med-Ox Diagnostics. Cellular fatty acid composition and library generation analysis were performed using the MIDI Sherlock system and LGS software (MIDI) as described previously (Bernard et al., 2002).

Cells of strain CCRI-16110T were organized as single cocci (0.5–1.0 μm) or in chains (Fig. 1). Some elongated oval cells could also be observed. The isolate grew well anaerobically, but no growth occurred following subculture to 5 % O₂, 5 % CO₂ or ambient air. Growth of the organism appeared to be enhanced by a fermentable carbohydrate (PYD) as well as with Tween 80, FF and bile, but it grew less efficiently in the presence of serum. After 3 days incubation at 37 °C under anaerobic atmosphere, colonies were 0.5–1.0 mm in diameter, convex and white in colour. No zone of haemolysis was observed on blood agar. Cells were not motile. Special-potency discs showed that the isolate was resistant to 1000 μg kanamycin, 10 μg colistin, 5 μg vancomycin and 5 % sodium polyanethol sulfonate. Susceptibility tests showed that the isolate was resistant to vancomycin (MIC >256 μg ml⁻¹) and teicoplanin (MIC >256 μg ml⁻¹), but susceptible to metronidazole (MIC <0.125 μg ml⁻¹). Results of chemotaxonomic analyses are given in the species description.

Total DNA from strain CCRI-16110T was purified with the GNO ME DNA kit (Qbiogene) according to the manufacturer’s instructions. PCRs to amplify an 884 bp region of the bacterial tuf gene encoding elongation factor Tu, which is involved in peptide chain formation (Ke et al., 2000), or a 1466 bp region of the 16S rRNA gene (Paradis et al., 2005) were performed as described previously (Ke et al., 1999). Sequencing of specific amplification products was also performed as described previously (Domingo et al., 2005a). To identify the taxonomic neighbours of strain CCRI-16110T, 16S rRNA gene sequences were used for an initial BLAST search against the GenBank database. Subsequently, bacterial species closely related to strain CCRI-16110T were used for phylogenetic analysis. Multiple sequence alignments were performed using CLUSTAL W from the GCG package (Wisconsin Package version 10.3; Accelrys). Phylogenetic analysis was carried out by the neighbour-joining (Saitou and Nei, 1987) and maximum-parsimony (Fitch, 1971) methods using MEGA version 4 (Tamura et al., 2007). Evolutionary distance matrices were...
The final 16S rRNA gene phylogenetic tree was rooted with *Fusobacterium nucleatum* ATCC 25586<sup>T</sup> and *Propionigenium modestum* Gra Succ 2<sup>T</sup> (cluster XIX) as the outgroup and bootstrap values were displayed as percentages.

Comparative 16S rRNA and *tuf* gene sequence analysis revealed that this unidentified isolate was closely related to members of cluster XIVa of *Clostridium* (Collins et al., 1994). Based on 16S rRNA gene sequence similarities, the closest relatives of the isolate were the type strains of *Clostridium bolteae* (93.8%), *Ruminococcus productus* (<93%), *Clostridium indolis* (<93%), *Clostridium asparagiforme* (<93%), *Clostridium saccharolyticum* (<93%) and *Ruminococcus hansenii* (92.8%). Neighbour-joining phylogeny using a larger number of 16S rRNA gene sequences (see Supplementary Fig. S1 available with IJSEM Online) showed that the unknown anaerobic Gram-positive coccus is a member of cluster XIVa of *Clostridium* (Collins et al., 1994). The maximum-parsimony method for 16S rRNA gene sequence analysis provided a similar tree topology (data not shown). Members of cluster XIVa that are Gram-positive cocci belong to the genera *Ruminococcus* and *Coprococcus* (Ezaki et al., 2006). The genus *Ruminococcus* is polyphyletic and distributed in cluster XIVa as well as in cluster IV of *Clostridium* (Ezaki et al., 2006). To elucidate the phylogenetic relationship between strain CCRI-16110<sup>T</sup> and Gram-positive cocci belonging to clusters XIVa and IV, phylogenetic trees based on 16S rRNA gene sequences were constructed using two different tree-making algorithms. The neighbour-joining (Fig. 2) and maximum-parsimony (Supplementary Fig. S2) trees both showed clearly that strain CCRI-16110<sup>T</sup> formed a distinct sublineage within the genus *Ruminococcus* belonging to cluster XIVa. However, bootstrap analysis of the 16S rRNA gene phylogenetic tree does not support a close association of this strain with any specific *Ruminococcus* species found within cluster XIVa (Supplementary Fig. S1). To ascertain the evolutionary position of the unknown bacterium within *Clostridium* cluster XIVa, a phylogenetic study based on *tuf* sequences from related taxa of this cluster was performed. The maximum-parsimony and neighbour-joining analysis with the *tuf* gene nucleotide and deduced amino acid sequences showed identical branching patterns for strain CCRI-16110<sup>T</sup> (data not shown). The phylogenetic tree based on the deduced amino acid of *tuf* gene sequences suggests that strain CCRI-16110<sup>T</sup> is most closely related to *R. productus* (Fig. 3).

Strain CCRI-16110<sup>T</sup> clearly represents a hitherto unknown bacterium from human faeces that fits into *Clostridium* cluster XIVa (Collins et al., 1994). Based on phylogenetic analysis of both 16S rRNA and *tuf* gene sequences, the unidentified bacterium is closely related to *Ruminococcus* species belonging to cluster XIVa. Species in the genus *Ruminococcus*, as found here with CCRI-16110<sup>T</sup>, have cocccoidal, Gram-positive forms arranged as chains, singles or diplococci. They use carbohydrates as fermentable substrates yielding acetic acid or other metabolic products such as succinic or lactic acid. Broth growth is stimulated by the presence of carbohydrate. They are catabolically fermentative, do not produce indole, are catalase- and oxidase-negative, do not reduce nitrates and are most frequently isolated from rumen, bowel (including human faeces) and caecum (Bryant, 1986). Strain CCRI-16110<sup>T</sup> is similar phenotypically to *R. productus* and *R. hansenii*, but may be distinguished from them by lactose and raffinose reactions (Bryant, 1986) (Table 1). Moreover, strain CCRI-16110<sup>T</sup> presents an important divergence in 16S rRNA gene

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**Fig. 2.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain CCRI-16110<sup>T</sup> (*R. gauvreauii* sp. nov.) and related taxa within *Clostridium* clusters XIVa and IV. The type strains of *Fusobacterium nucleatum* and *Propionigenium modestum* (*Clostridium* cluster XIX) were used as the outgroup. The tree was constructed using the neighbour-joining method based on a comparison of approximately 1105 nt. Bootstrap values, expressed as percentages of 1000 replications, are given at each branching point. GenBank accession numbers are given in parentheses.
sequence (>6%) from other known Ruminococcus species. Although it has been recommended that the geographical, phenotypic and genotypic diversity of at least five isolates should be used to describe a new taxon (Christensen et al., 2001), novel species containing a single strain can be validly named if their phenotype and genotype have been thoroughly and adequately characterized (Vandamme et al., 1996). Since no additional fastidious anaerobic bacterium identical to strain CCRI-16110^T has been isolated from faecal specimens, polyphasic taxonomy remains the best method to differentiate bacteria at the species level (Vandamme et al., 1996). In conclusion, it is proposed that this isolate represents a novel species, named Ruminococcus gauvreauii sp. nov., based on the findings presented here.

**Description of Ruminococcus gauvreauii sp. nov.**

*Ruminococcus gauvreauii* [gau.vreau’i.i. N.L. masc. gen. n. gauvreauii of Gauvreau, named after Leò Gauvreau (MD, FRCPC), a microbiologist Emeritus Professor, former Director of the Department of Microbiology of Université Laval in Quebec City (Québec, Canada), known as an excellent teacher for his contribution to clinical diagnostic microbiology especially as it relates to botulism, an infection often observed in aboriginal people from northern Canada].

Strictly anaerobic, Gram-positive cocci (0.5–1.0 \( \mu \)m). Cells are not motile. Colonies on Brucella blood agar are small, white and convex. Growth occurs at 35–37 °C under anaerobic conditions. Resistant to vancomycin and teicoplanin and harbours the acquired *vanD* gene cluster. Indole, catalase, oxidase, lecithinase, lipase and urease are not produced. Nitrate is not reduced to nitrite. Aesculin, gelatin and starch are not hydrolysed. Produces acid from D-glucose, D-galactose, D-fructose, D-ribose, D-sorbitol, D-mannitol, inositol and sucrose. Does not produce acid from D-lactose, maltose, D-mannose, trehalose, D-arabinose, cellobiose, \( \alpha \)-melibiose, \( \alpha \)-raffinose, L-xyllose, D-salicyl, adonitol, amygdalin, glycerol, glycogen, erythritol, inulin, \( \alpha \)-melezitose, L-rhamnose or starch. No peptonization of milk occurs. Biochemical tests using Rapid ID 32A and RapID ANA II systems are negative for all substrates tested. Based on testing with the API ZYM system, only acid phosphatase and naphthol-AS-BI-phosphohydrolase are produced. The major end product of glucose metabolism is acetic acid. The long-chain cellular fatty

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**Table 1.** Characteristics that are useful for distinguishing strain CCRI-16110^T (R. gauvreauii sp. nov.) from closely related anaerobic Gram-positive cocci within cluster XIVa

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of aesculin</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>V</td>
<td>d</td>
<td>NA</td>
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<tr>
<td>Acid from:</td>
<td></td>
<td></td>
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<tr>
<td>Cellobiose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>d</td>
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<tr>
<td>Raffinose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>End product(s) of glucose metabolism*</td>
<td>A, l, s</td>
<td>E</td>
<td>L, a, s</td>
<td>S, l</td>
<td>A, S, H, l</td>
<td></td>
</tr>
</tbody>
</table>

* A/a, Acetic acid; E, ethanol; H, hydrogen; L/l, lactic acid; S/s, succinic acid. Minor products are indicated by lower-case letters.
acids consist primarily of C_{16:0} (16.94 %), C_{14:0} (16.91 %), C_{18:1} cis9 dimethyl acetyl (DMA) (13.53 %), C_{18:1} cis11 DMA (9.94 %) and C_{18:1} cis9 (8.41 %), as well as other minor components of DMA forms including C_{14:0} DMA (5.96 %), C_{16:0} DMA (4.03 %), C_{16:1} cis9 DMA (3.95 %) and C_{18:0} DMA (2.64 %). Only acetic acid is produced as end product in PY medium.

The type strain is CCRI-16110T (=NML 060141T=CCUG 54292T =ICM 14987T), isolated from a human faecal specimen. The extent of habitat is not known, but is probably the mammalian gastrointestinal tract.

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References


