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

M.-C. Domingo,^{1,2} A. Huletsky,^{1,2} M. Boissinot,^{1,2} K. A. Bernard,^{3,4}
F. J. Picard^{1,2} and M. G. Bergeron^{1,2}

Correspondence

M. G. Bergeron
michel.g.bergeron
@crchul.ulaval.ca

¹Centre de Recherche en Infectiologie de l'Université Laval, CHUQ, Pavillon CHUL, 2705 boul. Laurier, Québec, QC, G1V 4G2, Canada

²Division de Microbiologie, Faculté de Médecine, Université Laval, Québec, QC, Canada

³National Microbiology Laboratory, Health Canada H5040-1015 Arlington St, Winnipeg, MB, Canada

⁴Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada

A novel strictly anaerobic, vancomycin-resistant, Gram-positive coccus (strain CCRI-16110^T) 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 -raffinose. 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-16110^T was most closely related to species of the genus *Ruminococcus* and formed a hitherto unknown sublineage within the *Clostridium coccoides* 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-16110^T (=NML 060141^T =CCUG 54292^T =JCM 14987^T).

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 coccoides* 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 *Coproccoccus* (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 μ l aliquot of a rectal swab suspension was cultured in brain-heart infusion (BHI) broth supplemented with 1 μ g vitamin K ml⁻¹ and 5 μ g haemin ml⁻¹ (eBHI) and incubated at 35 °C in an anaerobic atmosphere for 24 h. The eBHI culture was subcultured onto eBHI agar (eBHIA) containing 32 μ g 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 μ g vitamin K ml⁻¹, 5 μ g 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-16110^T 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.

L-cystine ml^{-1} , 10 mM sodium lactate, 10 mM sodium pyruvate, 10 μg vancomycin ml^{-1} and 100 μg aztreonam ml^{-1} (eBHILPVA broth) was used. Up to 11 subcultures in eBHILPVA broth separated by 3–7 days of incubation were performed at 35–37 °C inside an anaerobic chamber containing a gas mixture of 5 % H_2 , 10 % CO_2 and 85 % N_2 . Growth of the *vanD*-positive bacterium was monitored by the intensity of the PCR signal between the subcultures using the specific *vanD* primers described previously by our group (Domingo *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-16110^T) was further processed for identification.

Phenotypic identification tests of strain CCRI-16110^T 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 manufacturers' 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-16110^T 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 % O_2 , 5 % CO_2 or ambient air. Growth of the organism appeared to be enhanced by a fermentable

Fig. 1. Transmission electron micrograph of thin-sectioned cells of strain CCRI-16110^T (*Ruminococcus gauvreauii* sp. nov.). Bar, 1 μm .

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 an 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 $\mu\text{g ml}^{-1}$) and teicoplanin (MIC >256 $\mu\text{g ml}^{-1}$), but susceptible to metronidazole (MIC <0.125 $\mu\text{g ml}^{-1}$). Results of chemotaxonomic analyses are given in the species description.

Total DNA from strain CCRI-16110^T was purified with the GNOME 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-16110^T, 16S rRNA gene sequences were used for an initial BLAST search against the GenBank database. Subsequently, bacterial species closely related to strain CCRI-16110^T 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

generated according to the Kimura II parameter for nucleotide sequences (Kimura, 1980) and the Jones–Taylor–Thornton matrix for amino acid sequences (Jones *et al.*, 1992). Bootstrap values were calculated from 1000 resamplings. The final 16S rRNA gene phylogenetic tree was rooted with *Fusobacterium nucleatum* ATCC 25586^T and *Propionigenium modestum* Gra Succ 2^T (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^T 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^T 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^T (data not shown). The phylogenetic tree based on the deduced amino acid of *tuf* gene sequences suggests that strain CCRI-16110^T is most closely related to *R. productus* (Fig. 3).

Strain CCRI-16110^T 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^T, have coccoidal, 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^T 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^T presents an important divergence in 16S rRNA gene

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain CCRI-16110^T (*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.

Fig. 3. Phylogenetic tree based on deduced amino acid sequences of the *tuf* gene of strain CCRI-16110^T (*R. gauvreauii* sp. nov.) and some representative members of *Clostridium* cluster XIVa. *F. nucleatum* ATCC 25586^T (16S rRNA gene cluster XIX) was used as the outgroup. The tree was constructed using the neighbour-joining method based on a comparison of 270 aa positions. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. GenBank accession numbers for *tuf* gene sequences 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 Léo 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 µm). 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, α-melibiose, α-raffinose, L-xylose, D-salicin, adonitol, amygdalin, glycerol, glycogen, erythritol, inulin, α-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

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

Species: 1, *R. gauvreauii* sp. nov.; 2, *R. productus*; 3, *R. obeum*; 4, *R. hansenii*; 5, *R. flavefaciens*; 6, *R. luti*. Data for reference species were taken from Jousimies-Somer *et al.* (2002), Simmering *et al.* (2002), Song *et al.* (2003) and Bryant (1986). +, Positive; –, negative; d, 11–89% of strains positive; NA, not available; v, variable.

Characteristic	1	2	3	4	5	6
Hydrolysis of aesculin	–	+	–	v	d	NA
Acid from:						
Cellobiose	–	+	–	–	+	+
Lactose	–	+	+	+	d	+
Raffinose	–	+	+	+	–	+
End product(s) of glucose metabolism*	A	A, l, s	E	L, a, s	S, l	A, S, H, l

*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} *cis*9 dimethyl acetyl (DMA) (13.53 %), C_{18:1} *cis*11 DMA (9.94 %) and C_{18:1} *cis*9 (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} *cis*9 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-16110^T (=NML 060141^T=CCUG 54292^T=JCM 14987^T), isolated from a human faecal specimen. The extent of habitat is not known, but is probably the mammalian gastrointestinal tract.

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