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Clostridium lavalense sp. nov., a glycopeptide-resistant species isolated from human faeces

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Two vancomycin-resistant, strictly anaerobic, Gram-positive, rod-shaped, spore-forming organisms (strains CCRI-9842^T and CCRI-9929) isolated from human faecal specimens in Québec, Canada, and Australia were characterized using phenotypic, biochemical and molecular taxonomic methods. Pairwise analysis of the 16S rRNA gene sequences showed that both strains were closely related to each other genetically (displaying 99.2 % sequence similarity) and represented a previously unknown subline within the *Clostridium coccoides* rRNA group of organisms (rRNA cluster XIVa of the genus *Clostridium*). Strains CCRI-9842^T and CCRI-9929 used carbohydrates as fermentable substrates, producing acetic acid as the major product of glucose metabolism. The novel strains were most closely related to *Clostridium asparagiforme*, *Clostridium bolteae* and *Clostridium clostridioforme*, but morphological, biochemical and phylogenetic studies demonstrated that they represent a previously unidentified species of the genus *Clostridium*. This was confirmed by the unique cellular fatty acid composition of strains CCRI-9842^T and CCRI-9929. Therefore, on the basis of data from the polyphasic taxonomic analysis, it is proposed that strains CCRI-9842^T and CCRI-9929 represent a novel species of the genus *Clostridium*, for which the name *Clostridium lavalense* sp. nov. is proposed. The type strain is CCRI-9842^T (=CCUG 54291^T=JCM 14986^T=NML 03-A-015^T).

Accurate analysis of the microbial ecosystem in the human digestive tract is always a challenging topic for microbiologists because of the interactions between complex bacterial communities and host cells and also because of the implications for human health (Backhed *et al.*, 2005; Hooper *et al.*, 2002; Zoetendal *et al.*, 2006). The microbiota of the human gut has been investigated extensively, using both traditional culture methods and molecular techniques, and has been found to contain a large number of unknown species belonging to the 19 phylogenetic clusters of the genus *Clostridium* (Collins *et al.*, 1994; Eckburg *et al.*, 2005; Suau *et al.*, 1999). The bacteria most frequently detected in human faeces can be classified as belonging to three major groups. One group comprises the low-G+C, Gram-positive organisms (46–58 %), as follows: the *Clostridium coccoides–Eubacterium rectale* group (also known as cluster XIVa) (22.7–28 %), the *Clostridium leptum* group (also known as cluster IV) (21.1–25.2 %), the *Lactobacillus–Enterococcus* group (<0.1–1.8 %), the *Eubacterium cylindroides* group (1.1–1.4 %), the *Veillonella* group (<0.1–1.3 %) and the *Phascolarctobacterium* group (0.6 %). The next category consists of Gram-negative organisms (10–30 %): the *Bacteroides–Prevotella* group (8.5–28 %), the family *Enterobacteriaceae* (0.1–0.2 %) and the genus *Akkermansia* (1.3 %). Finally, there are the high-G+C, Gram-positive organisms (8–17 %) represented by the genera *Atopobium* (3.1–11.9 %) and *Bifidobacterium* (4.4–4.8 %) (Zoetendal *et al.*, 2006).

Two strains, CCRI-9929 (=NML 03-A-014=MLG245) and CCRI-9842^T (=NML 03-A-015^T) were isolated during

Abbreviation: MIC, minimum inhibitory concentration.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain CCRI-9842^T and CCRI-9929 are EF564277 and EF564278, respectively.

Neighbour-joining, maximum-parsimony and maximum-likelihood cladograms, based on 16S rRNA gene sequences of strains CCRI-9842^T and CCRI-9929 and related taxa, are available as supplementary figures with the online version of this paper.

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screening of human faecal specimens to detect carriers of vancomycin-resistant enterococci (Ballard *et al.*, 2005; Domingo *et al.*, 2005; Stinear *et al.*, 2001). In this study, we characterized the phenotypic profiles and phylogenetic relationships of these two strains. The data from this polyphasic taxonomic analysis, presented here, revealed that these strains represent a novel species of the genus *Clostridium*.

During a screening programme for the detection of vancomycin-resistant enterococci in clinical settings in Québec (Canada) and Melbourne (Australia), two anaerobic bacterial strains, CCRI-9929 and CCRI-9842^T, were isolated, respectively, from faecal specimens of patients tested for colonization by vancomycin-resistant enterococci (Ballard *et al.*, 2005; Domingo *et al.*, 2005; Stinear *et al.*, 2001). No further information was collected from these patients. The isolates were stored at −80 °C in brain heart infusion (BHI; Difco) +20 % glycerol media until the identification tests could be performed. These strains were subcultured twice on BHI blood agar containing haemin (0.005 mg ml^{-1}) and vitamin K (0.001 mg ml^{-1}). Phenotypic identification tests 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, Université Laval, Québec, Canada, and at the National Microbiology Laboratory, Winnipeg, Canada. Morphology was observed using light microscopy (Leitz) and transmission electron microscopy (1200EX; JEOL). Growth was observed in peptone-yeast broth enriched with glucose, with or without bile, serum, Tween 80 or formate-fumarate (Jousimies-Somer *et al.*, 2002). Special-potency discs (Oxoid) containing colistin (10 μg), kanamycin (1000 μg), vancomycin (5 μg) and metronidazole (5 μg) were used to group the anaerobic bacteria, as recommended in the Wadsworth manual (Jousimies-Somer *et al.*, 2002). Minimum inhibitory concentrations (MICs) for vancomycin and metronidazole were determined using the agar dilution method for anaerobes according to the Clinical and Laboratory Standard Institute (NCCLS, 2004). The MIC for teicoplanin was determined using the Etest method (AB Biodisk). Fermentation tests were performed for various sugars using prereduced, anaerobically sterilized peptone-yeast sugar broth tubes (Med-Ox Diagnostics). Other tests, such as those for catalase, aesculin, indole, lecithinase, lipase, nitrate and oxidase, were performed as described previously (Jousimies-Somer *et al.*, 2002). The novel isolates were examined (according to the manufacturers' instructions) using enzyme test kits including the Rapid ID 32A and API ZYM systems (bioMérieux) and the Rapid ANA II system (Remel). GLC analysis of the metabolic end products of fermentation was performed 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 analyses were performed using the MIDI Sherlock system and LGS software (MIDI), as described previously (Bernard *et al.*, 2002).

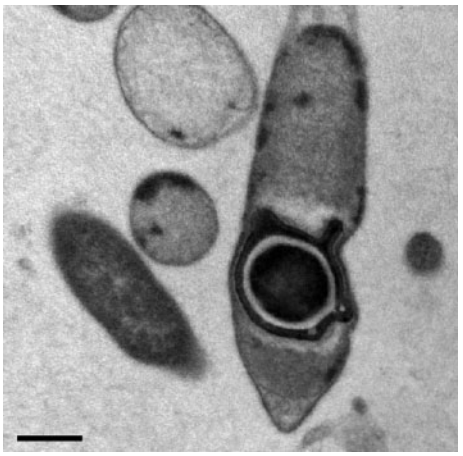

Fig. 1. Transmission electron micrograph of thin-sectioned cells of strain CCRI-9842^T. Bar, 0.5 μm.

Strains CCRI-9842^T and CCRI-9929 comprised strictly anaerobic, Gram-positive, rod-shaped cells (0.8–1.5 μm wide by 2.5–6.0 μm long) with tapered ends. Subterminal spores were observed (Fig. 1). The isolates grew well anaerobically, but no growth occurred following subculture under 5 % O_2. Growth was enhanced in the presence of glucose and formate-fumarate for both strains, and in the presence of bile and serum in the case of strain CCRI-9929 (Table 1). Neither strain was inhibited by Tween 80. After 48 h incubation on blood agar at 37 °C under anaerobic conditions, colonies of both strains were 2–3 mm in diameter, greyish white in colour and had irregular edges. The centres of the colonies were slightly raised and no zones of haemolysis were observed on blood agar. Colonies of strain CCRI-9842^T were strongly adherent on blood agar whereas those of strain CCRI-9929 were non-adherent on the same medium. This adherence of strain CCRI-9842^T declined progressively after several subcultures on agar medium. Cells of both novel isolates were motile. The isolates were indole-positive and catalase-negative. Nitrate was reduced by strain CCRI-9842^T but not by strain CCRI-9929. No liquefaction of gelatin or peptonization of milk occurred in either strain. Neither strain hydrolysed urea or starch. Analysis using special potency discs showed that both strains were susceptible to 1000 μg kanamycin and 5 μg metronidazole and that both were resistant to 10 μg colistin and 5 μg vancomycin. MIC determinations showed that both strains were resistant to vancomycin (MIC> 256 μg ml^{-1}) but were susceptible to teicoplanin (MIC< 1.5 μg ml^{-1}) and metronidazole (MIC<0.064 μg ml^{-1}). The enzyme profile obtained using Rapid ID 32A and API ZYM tests is summarized in Table 1. Both strains belong to the genus *Clostridium*, being obligatory anaerobic, endospore-forming, Gram-positive bacilli. On the basis of their cultural and biochemical characteristics, as well their glycopeptide susceptibility profile, it was evident that these

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Table 1. Characteristics useful for differentiating strain CCRI-9842^T from the type strains of closely related members of the genus *Clostridium*

Strains: 1, CCRI-9842^T; 2, *Clostridium asparagiforme* DSM 15981^T (data from Mohan *et al.*, 2006); 3, *Clostridium bolteae* WAL 16351^T (Song *et al.*, 2003); 4, *Clostridium clostridioforme* ATCC 25537^T (Mohan *et al.*, 2006). +, Positive; −, negative; W, weak reaction; NA, data not available.

Characteristic	1	2	3	4
Indole production	+	+	−	−
Acid from:				
D-Arabinose	W	−	+	+
Cellobiose	−	NA	NA	NA
D-Mannose	+	−	+	+
Raffinose	−	−	NA	+
D-Ribose	W	NA	−	+
D-Sorbitol	W	NA	+	−
L-Xylose	W	−	+	+
Enzyme activity				
α-Galactosidase	−	+	NA	NA
β-Galactosidase	+	+	+	−
α-Glucosidase	−	+	−	+
α-Fucosidase	−	+	NA	NA
Naphthol-AS-BI-phosphohydrolase	+	−	NA	NA
β-Glucuronidase	−	−	NA	NA
End products of glucose metabolism*	A, l, s	A, L, E, h, f	A, L	A, L, E

*A, acetic acid; E, ethanol; f, formate; h, hydrogen; L or l, lactate; s succinic acid. Minor end products are indicated by lower-case letters.

isolates closely resembled each other and probably belonged to the same species.

Total DNA from strains CCRI-9842^T and CCRI-9929 was purified using the GNOME DNA kit (Qbiogene). Purified genomic DNA was used in PCRs to amplify a 1466 bp region of the 16S rRNA genes as described previously (Paradis *et al.*, 2005). Sequencing of the specific amplification products was also performed as described previously (Domingo *et al.*, 2005). To identify the taxonomic neighbours of strains CCRI-9842^T and CCRI-9929, 16S rRNA gene sequences were used for an initial BLAST search against the GenBank database. Subsequently, bacterial species closely related to both strains were used for a phylogenetic analysis. Multiple sequence alignments were performed using CLUSTAL W from the GCG package (Wisconsin Package, version 10.3; Accelrys). The phylogenetic analysis was carried out using the neighbour-joining (Saitou and Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with MEGA, version 4 (Tamura *et al.*, 2007). Evolutionary distance matrices were generated according to the Kimura two-parameter model (Kimura, 1980). Bootstrap values (%) were calculated from 1000 resamplings. The final phylogenetic tree based on 16S rRNA gene sequences was rooted with type strains of *Fusobacterium nucleatum* and *Propionigenium modestum* (cluster XIX) as the outgroup. The comparative 16S rRNA gene sequence analysis showed that isolates CCRI-9842^T and CCRI-9929 were genetically very closely related to each other, displaying 99.2 % sequence similarity. Searches in the sequence database revealed that the novel isolates were closely related to members of cluster XIVa of the genus *Clostridium* (Collins *et al.*, 1994). On the basis of 16S rRNA gene sequence similarity, the closest relatives of the unidentified isolates were type strains of *Clostridium asparagiforme* (97 %), *Clostridium bolteae* (96 %) and *Clostridium clostridioforme* (95 %). A phylogenetic tree generated using a larger number of 16S rRNA gene sequences and constructed using the neighbour-joining and maximum-parsimony methods showed that the unknown anaerobic isolates fitted into the *Clostridium coccoides* rRNA complex (cluster XIVa of the genus *Clostridium*) (Collins *et al.*, 1994) (Fig. 2 and Supplementary Figs S1 and S2, available with IJSEM Online). Cluster XIVa contains various genera, including *Clostridium*, *Ruminococcus*, *Eubacterium*, *Acetitomaculum*, *Roseburia* and *Coprococcus* (Collins *et al.*, 1994). To elucidate the phylogenetic relationships between the novel isolates and the *Clostridium* species belonging to cluster XIVa, phylogenetic trees based on 16S rRNA gene sequences were constructed by using three different tree-making algorithms. The neighbour-joining (Fig. 2), maximum-parsimony (see Supplementary Fig. S3, available with IJSEM Online) and maximum-likelihood (Supplementary Fig. S4 in IJSEM Online) trees clearly demonstrated that the two novel isolates represent a hitherto unknown subline within cluster XIVa of the genus *Clostridium*. The isolates clustered within a distinct subgroup that included *Clostridium asparagiforme*, *Clostridium bolteae*, *Clostridium clostridioforme*, *Clostridium citroniae* and *Clostridium aldenense*. It is generally accepted that organisms displaying 16S rRNA gene sequence dissimilarities close to 3 % or more do not belong to the same species (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). Several characteristics, including morphology, biochemical profiles and the 16S rRNA gene sequence tree topology, were used to distinguish strains CCRI-9842^T and CCRI-9929 from the closely related species *Clostridium asparagiforme*. Strains CCRI-9842^T and CCRI-9929 were resistant to vancomycin and harboured the *vanB* gene cluster. The cellular morphology of these strains was different from the asparagus-shaped morphology of cells of *Clostridium asparagiforme* (Fig. 1). These novel isolates also differed from *Clostridium asparagiforme* in that they produced acid from arabinose, mannose and xylose (Table 1). Enzymic activities for the novel isolates differed from those of *Clostridium asparagiforme* DSM 15981^T: the novel isolates showed negative reactions for α-glucosidase, α-fucosidase, α-galactosidase and β-glucuronidase. For both novel isolates, acetic acid was the major end product, with minor yields of lactate and succinate from glucose metabolism; in contrast, acetic acid, lactate

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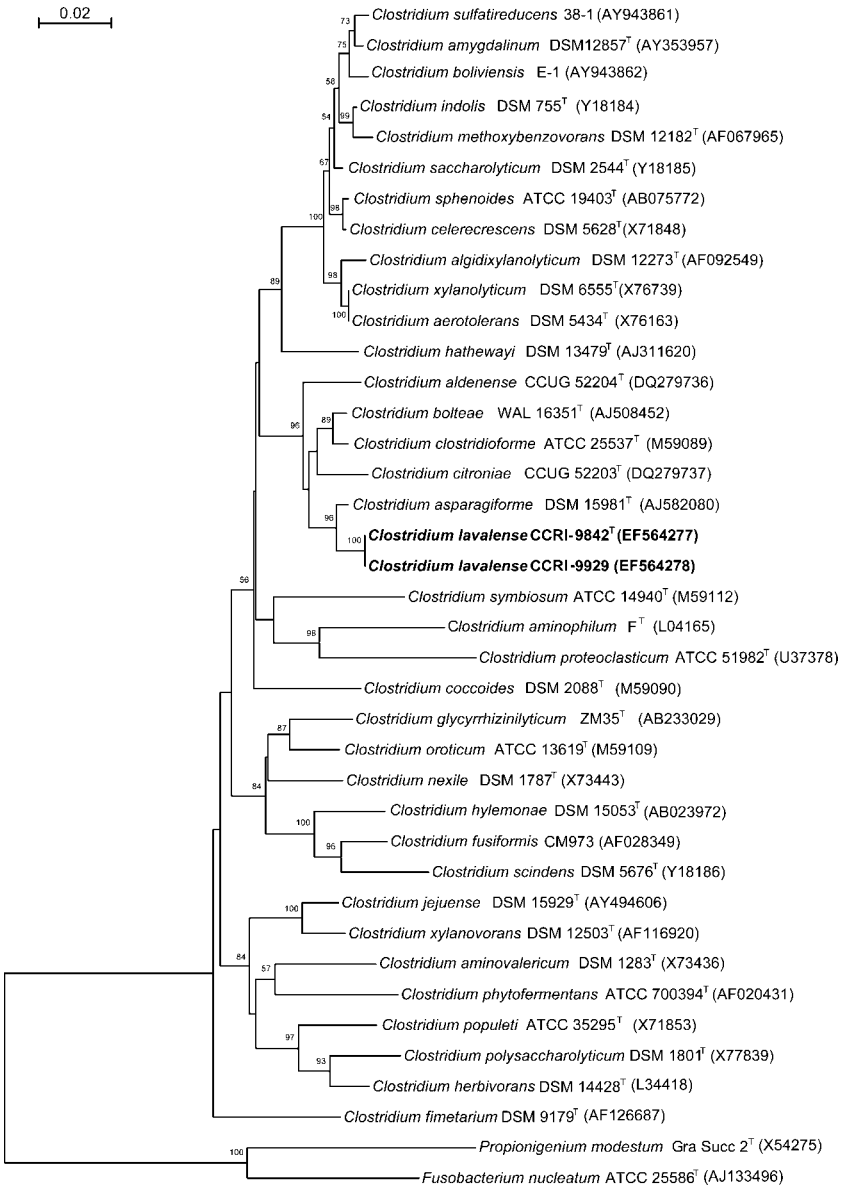

Fig. 2. Neighbour-joining phylogenetic tree, based on a comparison of partial (1118 nt) 16S rRNA gene sequences, showing the relationships of strains CCRI-9842^T and CCRI-9929 and related taxa within cluster XIVa of the genus *Clostridium*. *F. nucleatum* ATCC 25586^T and *P. modestum* Gra Succ 2^T (cluster XIX of *Clostridium*) were used as the outgroup. Bootstrap percentages (based on 1000 replications) are shown at branching points. GenBank accession numbers for each 16S rRNA gene sequence are given in parentheses. Bar, 0.02 substitutions per site.

and ethanol were the major end products for *Clostridium asparagiforme* DSM 15981^T. Long-chain cellular fatty acid profiles showed that strains CCRI-9842^T and CCRI-9929 differed from *Clostridium asparagiforme* DSM 15981^T in several respects (Table 2). Moreover, the branching pattern in the tree based on 16S rRNA gene sequences clearly shows that the novel bacterial isolates represent a separate species within cluster XIVa of the genus *Clostridium*. Despite the close relationships demonstrated to exist between the novel isolates and *Clostridium asparagiforme*, the data from the polyphasic taxonomic analysis presented here strongly support the classification of strains CCRI-9842^T and CCRI-9929 within a novel species of the genus *Clostridium*, for which the name *Clostridium lavalense* sp. nov. is proposed.

Description of *Clostridium lavalense* sp. nov.

Clostridium lavalense (la.va.len′se. N.L. neut. adj. *lavalense* pertaining to the institution, Université Laval, Québec, Canada).

Cells are strictly anaerobic, Gram-positive rods (0.8–1.5 μm in width and 2.5–6.0 μm in length) with tapered ends. Spores are subterminal (Fig. 1). Growth occurs under anaerobic conditions at 35–37 °C, but no growth occurs following subculture under a 5 % O_2 atmosphere. After 48 h incubation under anaerobic conditions, colonies are approximately 2 mm in diameter and greyish white in colour. Colony centres are slightly raised and the edges are rough. Zones of haemolysis are absent. The type strain, CCRI-9842^T, is strongly adherent to agar media. Acetic

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Table 2. Cellular fatty acid content (% of total) for strain CCRI-9842^T, *Clostridium asparagiforme*, *Clostridium bolteae* and *Clostridium clostridioforme*

Strains: 1, CCRI-9842^T; 2, *C. asparagiforme* DSM 15981^T; 3, *C. bolteae* DSM 15670^T; 4, *C. clostridioforme* DSM 933^T. Data for taxa 2–4 are from Mohan *et al.* (2006). NA, Data not available.

Fatty acid	1	2	3	4
$C_{16:0}$	27.96	14.84	12.6	25.16
$C_{18:1}$ *cis*9	10.32	5.77	6.1	4.41
$C_{18:1}$ *cis*11 DMA	8.80	NA	NA	NA
$C_{14:0}$	8.07	5.84	11.67	9.42
$C_{16:1}$ *cis*9	7.59	3.04	16.03	7.5
$C_{16:0}$ DMA	5.80	8.14	1.38	6.08
$C_{16:1}$ *cis*9 DMA	5.42	13.76	12.6	7.83
$C_{18:0}$	2.94	0.43	NA	NA
$C_{18:1}$ *cis*9 DMA	1.29	5.42	6.07	4.2
$C_{16:1}$ *cis*7	1.14	NA	NA	NA
$C_{16:0}$ ALDE	1.09	NA	NA	NA
$C_{12:0}$	0.63	0.33	0.64	0.43
$C_{18:0}$ 12-OH	0.55	NA	NA	NA
$C_{14:0}$ DMA	0.49	1.29	0.67	2.2
$C_{18:0}$ DMA	0.47	0.54	0.26	0.9
$C_{16:1}$ *cis*11	0.29	NA	NA	NA
$C_{14:1}$ *cis*7 DMA	0.23	0.73	0.71	0.49
$C_{10:0}$	0.21	NA	NA	NA
$C_{18:0}$ ALDE	0.18	0.41	1.39	0.47
$C_{20:1}$ *cis*11	0.17	NA	NA	NA
$C_{11:0}$ DMA	0.13	0.33	NA	0.22

acid is the major end product of fermentation. The stoichiometry of the fermentation products formed from glucose for the type strain is as follows: acetic acid, 22.1 mEq l^{-1}; lactic acid, 1.1 mEq l^{-1}; and succinic acid, 0.2 mEq l^{-1}. No other short-chain metabolic products are detected. Acid is produced from D-glucose, D-galactose, D-fructose, D-lactose, maltose, D-mannose, trehalose, sucrose and D-salicin. Acid is not produced from adonitol, amygdalin, glycerol, glycogen, inositol, erythritol, inulin, D-mannitol, α-melezitose, L-rhamnose or starch. Utilization of D-arabinose, cellobiose, D-ribose, α-melibiose, α-raffinose, D-sorbitol and L-xylose is variable (Table 1). Aesculin, gelatin and urea are not hydrolysed. Negative for lecithinase and lipase. Indole is produced. The predominant cellular fatty acids detected are $C_{16:0}$, $C_{18:1}$ *cis*9, $C_{18:1}$ *cis*11 DMA and $C_{14:0}$. Resistant to vancomycin (MIC>256 μg ml^{-1}) owing to the presence of the glycopeptide-resistance gene *vanB*.

The type strain, CCRI-9842^T (=CCUG 54291^T=JCM 14986^T=NML 03-A-015^T), was isolated from human faecal material. The extent of the habitat is not known, but is probably the mammalian gastrointestinal tract.

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