Peptostreptococcus russellii sp. nov., isolated from a swine-manure storage pit

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Using a polyphasic approach, a taxonomic study was performed on seven strains of an unknown Gram-reaction-positive, non-spore-forming, obligately anaerobic coccus-shaped bacterium, isolated from a swine-manure storage pit. Comparative 16S rRNA gene sequencing confirmed that all seven isolates were highly related to each other and formed a hitherto unknown lineage within the clostridial rRNA XI cluster of organisms. Pairwise analysis demonstrated that the novel organism was most closely related to *Peptostreptococcus anaerobius* CCUG 7835^T and *Peptostreptococcus stomatis* CCUG 51858^T with 16S rRNA gene sequence similarities of 95.5 and 93.0%, respectively. The peptidoglycan type of the cell wall was determined to be A4α L-Lys–D-Asp and glucose, xylose and traces of mannose were detected as the cell-wall sugars. Based on biochemical, chemotaxonomic and phylogenetic evidence the unknown bacterium represents a new species of the genus *Peptostreptococcus*, for which the name *Peptostreptococcus russellii* sp. nov. is proposed. The type strain is RT-10B^T (=CCUG 58235^T =NRRL B-59380^T =DSM 23041^T).

Lagoon treatment or deep pit storage methods, used to manage liquid swine manure, are associated with modern intensive livestock production. An understanding of the underlying processes of the production of odorous chemicals, including ammonia, organic acids, alcohols and sulphides by micro-organisms in manure is the focus of on-going research on swine manure management (Miller, 2001). During a continuing study of the microflora of underground manure storage pits, seven strains of a Gram-reaction-positive, strictly anaerobic, coccus-shaped bacterium were isolated (Whitehead & Cotta, 2004). Comparative 16S rRNA gene sequencing confirmed that these seven isolates were highly related to each other and formed a hitherto unknown lineage within the clostridial rRNA XI cluster of organisms (Collins *et al.*, 1994). Pairwise analysis demonstrated that the novel organism was most closely related to *Peptostreptococcus anaerobius* CCUG 7835^T and *Peptostreptococcus stomatis* CCUG 51858^T with 16S rRNA gene sequence similarities of 95.5 and 93.0%, respectively.

Abbreviation: HAP, hyper-ammonia-producing.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RT-10B^T is AY167952.

Two supplementary figures are available with the online version of this paper.

The genus *Peptostreptococcus* was created in 1936 by Kluyver and van Niel and by the 1990s contained 18 species that comprised obligately anaerobic, Gram-positive, non-sporulating, coccoid and coccobacillary bacteria that were physiologically and biochemically diverse (Ezaki, 2009). The advent of 16S rRNA sequencing confirmed the heterogeneity of the genus, which has subsequently undergone major revisions with many species being transferred to other genera, including *Anaerococcus*, *Peptoniphilus*, *Finegoldia*, *Micromonas*, *Gallicola*, *Slackia*, *Ruminococcus* and *Atopobium* (Ezaki, 2009; Murdoch *et al.*, 2000; Ezaki *et al.*, 2001). Indeed, after this reorganization, and until the recent description of the species *Peptostreptococcus stomatis*, isolated from the human oral cavity (Downes & Wade, 2006), *Peptostreptococcus anaerobius* remained the only species of the genus *Peptostreptococcus* with a validly published name. Based on phenotypic and phylogenetic evidence, it is proposed that the unknown bacterium, originating from underground swine manure pits, be classified as novel species of the genus *Peptostreptococcus* as *Peptostreptococcus russellii* sp. nov.

Seven isolates, designated RT-10B^T (=CCUG 58235^T =NRRL B-59380^T =DSM 23041), 37-1 (=CCUG 58233), RT-23A (=CCUG 58234), RT-18B, RT-19A, RT-8A and RT-13B, were recovered from a swine manure storage pit located near Peoria, IL, USA. The strains were

isolated as part of a study focusing on the identification of hyper-ammonia-producing (HAP) bacteria in swine manure (Whitehead & Cotta, 2004). The unidentified organisms were cultured on a basic medium containing macrominerals, microminerals, buffers, reducing agents and other components, as in the routine growth medium (RGM) described by Hespell *et al.* (1987), with 0.2 % carbohydrate added as a carbon source. All media used in these experiments were prepared anaerobically using the method of Hungate (1969) with modifications (Bryant, 1972). Media used for isolation of bacteria were based on RGM. Selection/Enrichment (SE) medium, containing buffer, salts, yeast extract (0.3 %) and 1 % Bacto-Tryptone (Becton Dickinson)/1 % Casamino Acids (vitamin-free, salt-free casein hydrolysate; ICN Biomedicals), was also used. SE agar plates were prepared in an anaerobic chamber (Coy Laboratory, Ann Arbor, MI, USA) in an atmosphere of 96 % CO₂:4 % H₂. Serial dilutions of manure samples were prepared with SE medium in the chamber. Aliquots (0.1 ml) of dilutions were plated directly onto SE-agar plates and incubated at room temperature (~24 °C), simulating the pit environment, or at 37 °C, until colonies were observed. The remaining dilutions were allowed to grow at either 24 °C or 37 °C for enrichment of peptide- or amino-acid-fermenting bacteria. The enrichment cultures were then streaked on SE-agar plates and incubated as before. All isolates were streaked several times on SE agar to obtain pure cultures. Growth of the isolates was also tested on Brain Heart Infusion (BHI) medium.

Gram-staining was performed with a commercial kit (Sigma) according to the manufacturer's instructions. Following the growth of the strains at 37 °C for 48 h on designated media, fermentation end-products were determined using HPLC. HPLC of organic acids was performed using an Aminex HPX-87H column (Bio-Rad) at 65 °C with 5 mM sulphuric acid as the solvent. Peaks were detected by using a model 410 differential refractometer (Waters) and identified by comparison with retention times of authentic standards (Cotta *et al.*, 2003). End-products of growth on RGM–glucose medium were (mM): acetate (13), lactate (2), formate (3), citrate (2) and trace amounts of succinate. End-products of growth on BHI medium were (mM): acetate (26), lactate (2), formate (2), citrate (7) and trace amounts of succinate. End-products of growth on SE medium were (mM): acetate (8.5), propionate (2.2), iso-butyrate (2.7), methyl-butyrate (2.6) and trace amounts of butyrate. Determination of DNA G+C contents (mol%) was carried out by thermal denaturation of chromosomal DNA using a Beckman model DU 640 spectrophotometer equipped with a high performance temperature controller and *T_m* analysis software (Johnson, 1994). The DNA G+C content of strain RT-10B^T was 35.6 mol%.

The unknown organisms recovered from the manure storage pit consisted of Gram-reaction-positive cocci, ~0.8–1.0 µm, occurring in pairs and chains of 3–10 cells. After 48 h of growth on BHI agar under anaerobic conditions, colonies were convex, 2–3 mm in diameter, opaque, smooth and whitish in colour. No growth was observed after 24 h of

incubation in static or shaking aerobic BHI broth or on aerobic BHI agar plates. The organism was able to grow in liquid culture at 25–45 °C (optimum 37 °C). No growth was observed at 4 or 50 °C. The strains were biochemically characterized by using the API rapid ID32An and API ZYM systems according to the manufacturer's instructions (bioMérieux). Using the API rapid ID32An system, only proline arylamidase activity was detected. All reactions in the API ZYM test system were negative. Nitrate was not reduced and indole was not produced. Presence of spores was determined by visual examination, as well as by incubation of cultures in 95 % ethanol followed by plating onto anaerobic agar medium. No spores were identified by either method. Coagulase activity was determined using a commercial kit (Sigma) according to the manufacturer's instructions with *Staphylococcus aureus* as a positive control. Strain RT-10B^T was found to be coagulase-negative and was also found to be catalase-negative using a slide test with 3 % hydrogen peroxide.

Long-chain cellular fatty acids were extracted and analysed by GC (MIDI) as described previously (Kämpfer & Kroppenstedt, 1996). The fatty acid composition of strain RT-10B^T is shown in Table 1 and revealed iso-C_{16:0} (17.3 %) as the only major (>10 %) fatty acid identified.

Analyses of the cell-wall sugars and peptidoglycan, respiratory quinones and polar lipids of strain RT-10B^T were carried out by the DSMZ Identification Service and Dr B.J. Tindall (DSMZ, Braunschweig, Germany) using the following methods. The tests were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose was used instead of paper chromatography. Quantitative analysis of amino acids in the peptidoglycan was performed using GC according to MacKenzie (1987). Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane (Tindall, 1990a, b) followed by phase separation into hexane. Polar lipids were extracted by adjusting the remaining methanol/0.3 % aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3 % aqueous NaCl mixture (1:2:0.8, v/v/v). The extraction solvent was stirred overnight and the cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3 % aqueous NaCl mixture to a ratio of 1:1:0.9 (v/v/v). Respiratory lipoquinones were separated into their different classes (e.g. menaquinones or ubiquinones) by TLC on silica gel (Macherey–Nagel, product ref: 805023), using a hexane/*tert*-butylmethylether mixture (9:1 v/v) as a solvent. UV absorbing bands corresponding to the different quinone classes were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical HPLC (Thermo Separation Products) fitted with a reversed-phase column (Macherey–Nagel; 2 × 125 mm, 3 µm, RP18) using a methanol/heptane 9:1 (v/v) mixture as the eluant.

Table 1. Cellular fatty acid compositions (%) of strain RT-10B^T and type strains of *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*

Strains: 1, RT-10B^T; 2, *Peptostreptococcus anaerobius* CCUG 7835^T; 3, *P. stomatis* CCUG 51858^T. All data were obtained in this study at the CCUG. All growth conditions were identical. Bold values represent major components. DMA, dimethyl acetal; –, not detected.

Fatty acid	1	2	3
C _{10:0}	–	1.1	–
anteiso-C _{11:0}	0.8	–	–
C _{12:0}	–	3.5	–
iso-C _{12:0}	5.7	4.5	0.4
iso-C _{13:0} 3-OH	–	2.4	5.5
anteiso-C _{13:0}	2.7	1.8	–
C _{14:0}	–	7.0	0.5
C _{14:0} 2,4 dimethyl	1.5	–	1.8
iso-C _{14:0}	1.2	5.5	23.3
C _{14:0} 2-OH	–	0.5	–
anteiso-C _{15:0}	–	1.4	0.5
C _{16:0}	2.2	19.3	7.7
C _{16:0} alde	–	–	0.9
iso-C _{16:0}	17.3	4.4	11.2
iso-C _{16:0} DMA	6.5	2.8	7.6
iso-C _{17:1} /C _{16:0} DMA	9.2	7.5	3.5
anteiso-C _{17:0}	4.4	1.0	1.7
anteiso-C _{17:0} DMA	2.1	–	–
C _{18:0}	2.2	1.2	3.3
iso-C _{18:0}	2.6	–	1.8
C _{18:0} ω9c	2.1	17.8	2.8
C _{18:1} ω9c DMA	2.9	5.4	4.2
C _{18:2} ω6,9c / anteiso-C _{18:0}	–	4.5	3.9
C _{18:2} DMA	4.8	5.4	6.6
iso-C _{19:1}	1.8	–	1.8
Unknown	9.7	11.9	7.9
Unidentified	3.8	2.5	2.3

Respiratory lipoquinones were detected at 269 nm. Polar lipids were separated by two-dimensional silica gel TLC (Macherey–Nagel, product ref: 818135). The first dimension was developed in chloroform/methanol/water (65:25:4, v/v/v), and the second in chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). Total lipid material was detected using dodecamolybdophosphoric acid and specific functional groups were detected with Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff reagent (α-glycols), Dragendorff (quaternary nitrogen) and α-naphthol-sulphuric acid (glycolipids). Analysis of cell-wall murein hydrolysates of strain RT-10B^T revealed the presence of peptides L-Ala–D-Glu, L-Lys–D-Ala and D-Ala–L-Lys–D-Asp, identifying it as having peptidoglycan type A4α L-Lys–D-Asp. Whole-cell-wall sugars of strain RT-10B^T consisted of glucose, xylose and traces of mannose. No respiratory quinones were detected. The polar lipid profile comprised aminoglycolipid, diphosphatidylglycerol, glycolipids, phosphatidylglycerol and phospholipids (Supplementary Fig. S1, available in IJSEM Online).

16S rRNA gene fragments were generated by PCR and sequenced as described previously (Whitehead & Cotta, 2004). The closest known relatives of the novel isolates were determined by performing database searches using the program FASTA (Lipman & Pearson, 1985). These sequences and those of related strains were retrieved from GenBank and aligned with the newly determined sequences using the program SEQtools (Rasmussen, 2002). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (Nicholas *et al.*, 1997), and a phylogenetic tree was reconstructed according to the neighbour-joining method (Saitou & Nei, 1987) using the programs SEQtools and TreeView (Page, 1996). The stability of the groupings was estimated by bootstrap analysis (based on 1000 replications) using the same programs. All major branching nodes were confirmed by maximum-parsimony analysis (data not shown). The seven isolates were highly related to each other genetically (99.4–100 % 16S rRNA gene sequence similarity). Sequence searches of DNA database libraries revealed that the unknown organism was a member of the phylum *Firmicutes* within the rRNA XI cluster of organisms (Collins *et al.*, 1994). Pairwise comparisons based on almost full-length 16S rRNA gene sequences (1400 nt) revealed significant sequence divergence between the unknown bacterium and its closest relatives, *Peptostreptococcus anaerobius* CCUG 7835^T and *Peptostreptococcus stomatis* CCUG 51858^T with 95.5 and 93.0 % sequence similarity, respectively. A phylogenetic tree, reconstructed using the neighbour-joining method, depicting the phylogenetic positions of strain RT-10B^T and related taxa is shown in Fig. 1.

After revision of the genus, *Peptostreptococcus anaerobius* remained the only species with a validly published name belonging to the genus *Peptostreptococcus*, demonstrating a distinct lineage from the other species and producing a range of volatile fatty acids as end-products of metabolism (Murdoch *et al.*, 2000; Ezaki, 2009). Downes & Wade (2006) described a second species of this amended genus, *Peptostreptococcus stomatis*, which was isolated from the human oral cavity, and demonstrated that in addition to phenotypic differences it possessed an additional 25 bases in the 16S rRNA secondary structure of the variable region 1 when compared with *Peptostreptococcus anaerobius*. Examination of the sequence of the novel strain RT-10B^T, isolated from swine manure, showed that these additional nucleotide bases were not present (Supplementary Fig. S2). There is no precise value of 16S rRNA sequence divergence which denotes delineation of bacterial species, but it is generally recognized that divergence values of ≥3 % are significant (Stackebrandt & Goebel, 1994) and recent data demonstrates that this value can be decreased to 1.3 % without loss of resolution (Stackebrandt & Ebers, 2006). Tree topology and high sequence divergence values (>4.5 %) suggested that strain RT-10B^T represents a novel species. The novel organism described here is phylogenetically located within the clostridial rRNA XI cluster of organisms, representing a unique line of descent within the genus *Peptostreptococcus*. In addition to sequence information, the

Fig. 1. Unrooted tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain RT-10B^T and other organisms within rRNA clusters XI and XIII. The tree was reconstructed using the neighbour-joining method and based on a comparison of ~1320 nt. Bar, 1 % sequence divergence.

novel organism contained D-aspartate within the interpeptide bridge of its peptidoglycan, like the type strain of *Peptostreptococcus anaerobius*. Likewise, strain RT-10B^T produced a number of end products from glucose metabolism, which included acetate, as the major component, smaller amounts of lactate, formate and citrate, and trace amounts of succinate. On the basis of direct plating and enrichment cultures, the novel isolates were present at concentrations of at least 10⁸ cells ml⁻¹, or approximately 0.1–1 % of the culturable bacterial population present in the swine manure samples. These strains produced prodigious amounts of ammonia (>40 mM) from various nitrogen sources (Tryptone and Casamino acids) and were termed HAP (Whitehead & Cotta, 2004). Therefore, this group of organisms may play an important role in the digestion and fermentation of proteinaceous material in the manure and the production of ammonia and other compounds (Attwood *et al.*, 1998; Whitehead & Cotta, 2004). The fermentation of amino acids such as tryptophan, phenylalanine and tyrosine are of particular interest and may give rise to the production of indole and phenolic compounds (such as skatole), contributing to the foul odours associated with swine facilities. Improved knowledge of these organisms should lead to a better understanding of the mechanisms involved and the development of strategies to combat deleterious microbial processes. Based on phenotypic, chemotaxonomic and phylogenetic evidence, the seven novel isolates, including strain RT-10B^T, represent a novel species of the genus *Peptostreptococcus*, for which the name *Peptostreptococcus russellii* sp. nov. is proposed. In addition to its unique 16S sequence, *Peptostreptococcus russellii* sp. nov. could be differentiated from *Peptostreptococcus anaerobius* CCUG 7835^T and *Peptostreptococcus stomatis* CCUG 51858^T by the characteristics shown in Table 1 and Table 2.

Description of *Peptostreptococcus russellii* sp. nov.

Peptostreptococcus russellii (rus.sel'li.i N.L. gen. masc. n. *russellii* of Russell, named after the late American microbiologist James B. Russell for his contributions to rumen microbiology and initial isolation of HAP bacteria).

The description is based on seven strains, isolated from swine faeces and stored manure. Cells are obligatory anaerobic, Gram-reaction-positive, non-motile cocci, ~0.8–1.0 µm,

occurring in pairs and chains of 3–10 cells. After 48 h of growth on BHI agar under anaerobic conditions, colonies are convex, 2–3 mm in diameter, opaque, smooth and whitish in colour. Grows in liquid culture at 25–45 °C (optimum 37 °C) but not at 4 or 50 °C. No growth occurs after 24 h in static or shaking aerobic BHI broth or on aerobic BHI agar plates. Weakly saccharolytic. Using the API rapid ID32An system: positive for proline arylamidase and negative for *N*-acetyl-β-glucosaminidase, alanine arylamidase, alkaline phosphorylase, arginine dihydrolase, α-arabinosidase, arginine arylamidase, α-fucosidase, α- and β-galactosidase, β-galactosidase-6-phosphate, α- and β-glucosidase, β-glucuronidase, glutamyl glutamic acid arylamidase, glycine arylamidase, histidine arylamidase, leucyl glycine arylamidase, mannose, phenylalanine arylamidase, pyroglutamic acid arylamidase, raffinose, serine arylamidase, tyrosine arylamidase and urease. Using the API ZYM identification system, no positive reactions are obtained. The major end-product from glucose metabolism is acetate, with smaller amounts of lactate, formate and citrate and trace amounts of succinate detected. Prodigious amounts of ammonia (>40 mM) are produced from various nitrogen sources (Tryptone and Casamino acids). Nitrate is not reduced and catalase, coagulase and indole are not produced. The cell-wall

Table 2. Differential characteristics of strain RT-10B^T and the type strains of *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*

Strains: 1, RT-10B^T; 2, *Peptostreptococcus anaerobius* CCUG 7835^T; 3, *P. stomatis* CCUG 51858^T. +, Positive; –, negative; w, weakly positive. α-Glucosidase and proline arylamidase activities were determined with the Rapid ID32A identification panel and hippurate was determined with the API ZYM identification panel (bioMérieux). Major fatty acid constitutes >10 % of total fatty acids.

Characteristic	1	2	3
Rapid ID32A profile	0000 0200 00	0400 0200 00	0400 0000 00
α-Glucosidase	–	+	+
Proline arylamidase	+	+	–
Hippurate	–	w	–
Major fatty acid(s)	iso-C _{16:0}	C _{16:0} , C _{18:1ω9c}	iso-C _{16:0} , iso-C _{14:0}

peptidoglycan is type A4 α L-Lys-D-Asp. Whole-cell-wall sugars consist of glucose, xylose and traces of mannose. The major long-chain fatty acid is iso-C_{16:0}. No respiratory quinones are detected. The polar lipid profile comprises aminoglycolipid, diphosphatidylglycerol, glycolipids, phosphatidylglycerol and phospholipids.

The type strain, RT-10B^T (=CCUG 58235^T =NRRL B-59380^T =DSM 23041^T), was isolated from a swine manure storage pit (habitat not known). The G + C content of the DNA of the type strain is 35.6 mol%.

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