Actinomyces ruminicola sp. nov., isolated from cattle rumen

Dengdi An, Shichun Cai and Xiuzhu Dong

Correspondence

Xiuzhu Dong

dongxz@sun.im.ac.cn

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, PR China

Two obligate anaerobic bacterial strains, B71^T and D471, were isolated from cattle rumen. The novel strains were Gram-positive and rod-shaped. The strains hydrolysed xylan and starch, fermented some mono-, di- and oligosaccharides and produced formic, acetic and lactic acids as end products from glucose. Growth of the isolates was observed at 20–55 °C and pH 6.5–9.0. The DNA G + C contents of strains B71^T and D471 were 68.06 and 68.26 mol%, respectively. Although the two novel strains met the genus description for *Actinomyces*, some phenotypic characteristics, such as optimum growth temperature, requirement for O₂ and the end products of fermentation, distinguished them from previously described members of the genus. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that the novel strains belonged to the genus *Actinomyces* (88.3–93.6 % sequence similarity) and formed a distinct line within the clade containing *Actinomyces bovis*. On the basis of these results, a novel species, *Actinomyces ruminicola* sp. nov., is proposed. The type strain is B71^T (=JCM 13352^T = CGMCC 1.5030^T).

The genus *Actinomyces* (Schaal, 1986) is a group of Gram-positive, asporogenous, non-acid-fast bacteria and cells are irregular rods, filamentous, diphtheroidal or branching. Species of the genus are either facultative or obligate anaerobes. Members of the genus *Actinomyces* primarily inhabit the mucosal surfaces of human and animals, such as the oral cavity (Schaal, 1986, 1992). In recent years, several novel species of *Actinomyces* have been isolated from human and animal sources (Hall *et al.*, 2003a, b, c, 2005; Hoyle *et al.*, 2001a, b, c, 2002; Lawson *et al.*, 2001; Nikolaitchouk *et al.*, 2000; Pascual *et al.*, 1999). While most of these species have been associated with clinical materials, their role in pathogenesis is not known and, at best, these organisms can be described as opportunistic pathogens.

Ruminants harbour numerous micro-organisms in their rumen that endow them with the ability to digest fibrous materials (Chesson & Forsberg, 1997; Koike *et al.*, 2003; Nelson *et al.*, 2003). During a study of rumen micro-organism composition, 10 bacterial strains that degraded xylan efficiently were obtained from a 4-year-old animal of the Jinnan cattle breed (*Bos taurus*), a local species from northeast China. Two of the novel strains were found to be closely related, phylogenetically and phenotypically, to members of the genus *Actinomyces*. However, both novel strains differed from recognized species. In this paper, a novel species of the genus *Actinomyces* is described.

Rumen content, sampled from a 4-year-old castrated bullock within 20 min after slaughter, was inoculated in xylan medium as described by Krause *et al.* (2001) and incubated under 100 % N₂ in anaerobic tubes (18 × 180 mm) sealed with butyl rubber stoppers at 39 °C for 3 days. After subculturing on xylan medium five times, strains B71^T and D471 were isolated from the xylan-degrading mixtures by the Hungate roll tube technique (Hungate, 1969) using the same medium containing 15 % agar. Single colonies were picked and transferred to PYRG broth [peptone-yeast extract-glucose (PYG) + 15 % rumen fluid]. Strains were routinely cultured in PYRG medium unless otherwise indicated.

Cell morphology was examined with a light microscope (BH-2; Olympus) and an electron microscope (H-600A; Hitachi). For electron microscopy, bacterial cells grown at 39 °C for 2 days were coated with a palladium/iridium alloy using a high vacuum evaporator (HUS-5GB; Hitachi). For determination of the end products of glucose fermentation, the novel strains were grown in PYRG at 39 °C for 7 days. Short-chain fatty acids were measured by GC (GC-14B; Shimadzu) using stainless steel columns packed with Porapak GDX-401 (60–80 mesh) and detected with a flame-ionization detector. For measurement of non-volatile fatty acids, methyl esters were derived from samples according to Holdeman *et al.* (1977) before analyses. Column temperatures were 220 and 150 °C for the measurement of volatile and non-volatile fatty acids, respectively. Nitrogen was used as the carrier gas in all analyses. The presence of formic acid was determined using HPLC (1525; Waters)

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Actinomyces ruminicola* strains B71^T and D471 are DQ072005 and DQ072006, respectively.

installed with a Hewlett Packard C₁₈ HPLC column (3.9 × 150 mm) according to Akalin *et al.* (2002). Acetonitrile (60 %, v/v) in ultra-purified water was used as the mobile phase and the flow speed was 0.3 ml min⁻¹.

The diagnostic isomers of diaminopimelic acid in the cell-wall peptidoglycan were determined using established TLC procedures (Lechevalier & Lechevalier, 1980). Cellular fatty acids were extracted, methylated and analysed using the standard MIDI system (Miller, 1982; Sasser, 1990). Men-aquinones were analysed by using HPLC (Kroppenstedt, 1985) with *Micromonospora halophytica* DSM 43171^T as a reference.

Temperature profiles were determined in PYRG using a water bath at temperatures of 15–55 °C at 1 °C intervals. The pH range for growth was determined in PYRG broth at various pH values adjusted with HCl or NaOH (1 M l⁻¹) at 39 °C. Growth was monitored by measuring the OD₆₀₀ of cultures at 1, 3 and 7 days. The generation time of the strains was determined by monitoring the OD₆₀₀ of the PYRG culture at 46 °C and pH 8.5 at 1 h intervals up to 48 h. Biochemical traits were assessed using conventional methods (Holdeman *et al.*, 1977). For determination of xylan degradation, 100 µl xylan culture was dried at 100 °C in a tube with a lid and then hydrolysed into monosaccharide by the addition of 500 µl 0.25 M H₂SO₄ at 120 °C for 90 min. The hydrolysed mixture was adjusted to neutral pH with 100 µl 10 M NaOH and the reductive carbohydrate was estimated as described by Miller (1959). Starch was determined by using the Congo red overlay method (Ruijsenaars & Hartmans, 2001). All tests were performed in duplicate.

Genomic DNA was extracted and purified using the method of Marmur (1961). The G+C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962) using a spectrophotometer (DU800; Beckman) with *Escherichia coli* K-12 as the reference. The 16S rRNA gene was amplified by PCR using a pair of universal primers, 27F (5'-AGAGTTTGATCC/ATGGC-TCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'), corresponding to base positions 8–27 and 1541–1525 of the 16S rRNA gene of *E. coli* (Winker & Woese, 1991), respectively. Extracted genomic DNA was used as a template and PCR amplification was performed with Thermolyne Amplitron I (Barnstead Thermolyne). PCR products were purified using a UNIQ-10 PCR product purification kit (Sangon) and ligated into vector pUCm-T (Sangon), as recommended by the manufacturer. Primers T7 (5'-GTAATACGACTCACTATAGG-3') and M13R (5'-CAGGAAACAGCTATGACCAT-3') were used to sequence the 16S rRNA gene fragment. Sequencing was performed by the Sangon Biological Engineering Technology Service, Shanghai, China, using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kits (Perkin Elmer) and an ABI PRISM 377XL DNA sequencer. The 16S rRNA gene sequences of strains B71^T and D471 were submitted to GenBank to search for similar sequences using the BLAST algorithm. The best matching sequences were retrieved from

the database and aligned. Similarity analysis was performed using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining and maximum-parsimony methods implemented in the MEGA2 (Kumar *et al.*, 2001) and PHYLIP software packages (Felsenstein, 1993). The resulting tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

Strains B71^T and D471 were Gram-positive, non-motile, non-spore-forming, straight rods (0.5–1.0 × 2.5–4.0 µm) and were non-acid-fast. They did not produce catalase and showed strictly anaerobic growth. The novel strains hydrolysed xylan and starch and fermented several kinds of mono-, di- and oligosaccharides. The major end products from glucose fermentation were formic, acetic and lactic acids. The novel strains did not hydrolyse hippurate or urea. Hydrolysis of aesculin and glycogen and fermentation of inositol, mannitol, mannose and salicin were variable between the two isolates. Both novel strains grew at 20–55 °C and at pH 6.5–9.0, with optimum growth at 46 °C and pH 8.0–8.5. The mean generation time for the novel strains was 2.21 h when grown in PYRG at 46 °C and pH 8.5.

To determine the phylogenetic position of the novel strains, their complete 16S rRNA gene sequences were analysed. A phylogenetic tree was constructed based on the similarity of a consensus length of 1367 bp of 16S rRNA gene sequence (Fig. 1). Strains B71^T and D471 were found to cluster with other species of the genus *Actinomyces*. However, the novel strains formed a distinct branch with relatively low sequence similarities (88.3–93.6 %) to other *Actinomyces* strains. The greatest gene sequence similarity (93.6 %) was shown with *Actinomyces denticolens* NCTC 11490^T (Dent & Williams, 1984b). Therefore, based on sequence divergence and phylogenetic evidence, it is clear that isolates B71^T and D471 represent a novel species of the genus *Actinomyces*.

The major cellular fatty acids of strain B71^T were mainly straight-chain saturated and monounsaturated fatty acids, with C_{16:0} (34.64 %), C_{18:0} (15.91 %) and C_{18:1ω9} (25.33 %) predominating. This fatty acid profile was different from those of phylogenetically related species such as *Actinomyces oricola* (Hall *et al.*, 2003c), *Actinomyces israelii* (Johnson *et al.*, 1990) and *Actinomyces bovis* (Johnson *et al.*, 1990) in which the major fatty acids are C_{16:0} (44, 38.9 and 23.7 %, respectively), C_{18:1 cis9} (37, 33.4 and 56.2 %, respectively) and C_{18:0} (14, 2.5 and 10.1 %, respectively). The fatty acid content of strain B71^T demonstrated the relationship of the novel strains to the genus *Actinomyces*. Strain B71^T contained menaquinones as the sole respiratory quinones, with MK-10 (70 %) as the major component and a low content of MK-9 (30 %).

The novel strains isolated from rumen contents could degrade xylan; a feature of their natural niche. The novel strains were readily distinguished from other species of the genus *Actinomyces* on the basis of phenotypic characteristics

Fig. 1. Neighbour-joining phylogenetic dendrogram of *Actinomyces ruminicola* sp. nov. strains B71^T and D471 and related species based on 16S rRNA gene sequence similarity. The tree was rooted with *Cellulomonas humilata* ATCC 25174^T. Solid circles indicate that the nodes were also recovered in maximum-parsimony and bootstrapping methods. Numbers at nodes are bootstrap values (%) based on neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 0.5% sequence divergence.

(Table 1). They differed from most of the other recognized *Actinomyces* species by showing strictly anaerobic growth and by having a higher optimum temperature for growth (46 °C). Other distinguishing characteristics were starch hydrolysis, sorbitol, rhamnose, cellobiose and D-arabinose fermentation and the formation of different fermentation products from glucose (Coleman *et al.*, 1969; Collins *et al.*, 2000; Dent & Williams, 1984a, b, 1986; Hall *et al.*, 2005, 2003c; Hoyles *et al.*, 2001b; Johnson *et al.*, 1990; Nikolaitchouk *et al.*, 2000; Pascual *et al.*, 1999; Schaal, 1992; Stackebrandt & Charfreitag, 1990) (see Table 1). The novel strains differed from *A. denticolens* in their natural niche, cell morphology, nitrate reduction and DNA G+C contents. Based on the phenotypic and phylogenetic distinctiveness of the rumen isolates, a novel species, *Actinomyces ruminicola* sp. nov. is proposed.

Description of *Actinomyces ruminicola* sp. nov.

Actinomyces ruminicola [ru.min.i'co.la. L. n. *rumen* -inis first stomach of ruminants, rumen; L. suff. -cola from L. n. *incola* an inhabitant, dweller; N.L. n. (nominative in apposition) *ruminicola* an inhabitant of rumen, indicating that the type strain was originally isolated from cattle rumen].

Cells are non-motile, straight rods (0.5–1.0 × 2.5–4.0 µm) that are Gram-positive, non-spore-forming, catalase-negative and strictly anaerobic. Colonies on PYRG agar are circular, slightly convex, white and reach approximately 0.5–0.8 mm in diameter after 48 h incubation at 46 °C. Formic acid is the main end product of glucose fermentation; acetate and lactate are also produced. Acids are produced from D-galactose, D-maltose, D-fructose (weak reaction), D-lactose, ribose, sucrose, erythritol, D-arabinose, D-xylose, cyclodextrin, rhamnose, sorbitol, cellobiose, melibiose, trehalose and melezitose. Acid production is variable from inositol, mannitol, mannose, salicin, glycogen and xylitol. Acid is not produced from glycerol, dulcitol, inulin, glucoside or adonitol. Hippurate and urea are not hydrolysed. Milk is curdled. Aesculin hydrolysis is variable. Nitrate is reduced. Methyl red test is positive, but Voges-Proskauer test is negative. The cellular fatty acids are of the straight-chain saturated and monounsaturated types, with C_{16:0}, C_{18:0} and C_{18:1ω9} predominating. The major respiratory quinones are menaquinones MK-10 (70%) and MK-9 (30%).

The type strain, B71^T (=JCM 13352^T =CGMCC 1.5030^T), was isolated from cattle rumen.

Table 1. Characteristics that differentiate *Actinomyces ruminicola* sp. nov. B71^T from other phylogenetically related species of the genus *Actinomyces*

Strains: 1, *Actinomyces ruminicola* sp. nov. strain B71^T; 2, *A. ruminicola* strain D471; 3, *A. denticolens* NCTC 11490^T (data from Dent & Williams, 1984b); 4, *Actinomyces dentalis* CCUG 48064^T (Hall *et al.*, 2005); 5, *A. oricola* CCUG 46090^T (Hall *et al.*, 2003b); 6, *Actinomyces catuli* CCUG 41709^T (Hoyle *et al.*, 2001b); 7, *A. israelii* CIP 103259^T (Stackebrandt & Charfreitag, 1990); 8, *A. bovis* ATCC 13683^T (Schaal, 1992); 9, *Actinomyces bowdenii* CCUG 37421^T (Pascual *et al.*, 1999); 10, *Actinomyces urogenitalis* CCUG 38702^T (Nikolaitchouk *et al.*, 2000); 11, *Actinomyces radicidentis* CCUG 36733^T (Collins *et al.*, 2000); 12, *Actinomyces howellii* ATCC 43323^T (Dent & Williams, 1984a); 13, *Actinomyces gerencseriae* ATCC 23860^T (Johnson *et al.*, 1990); 14, *Actinomyces naeslundii* (Coleman *et al.*, 1969); 15, *Actinomyces viscosus* ATCC 15987^T (Johnson *et al.*, 1990); 16, *Actinomyces slackii* ATCC 49928^T (Dent & Williams, 1986). +, Positive; –, negative; W, weakly positive; V, variable; Fan, facultatively anaerobic; An, anaerobic; Ae, aerobic; Fil, filamentous; ND, not detected, NP, not reported. All experiments in this study were carried out under strictly anaerobic conditions.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Origin	Rumen	Rumen	Cattle	Human teeth	Human teeth	Dogs	Human	Bovine	Canine, feline	Human, clinical	Human teeth	Cattle teeth	Human	Human sinus	Human	Cattle teeth
Cell shape	Rod	Rod	Rod	Fil	Rod/Fil	Rod	Fil	Rod	Rod	Rod	Cocci	Rod	Fil	Rod	Rod	Rod
Relation to O ₂	An	An	Fan	An	Fan	Fan	Fan	Fan	Fan	Fan	Fan	Fan	An	Fan	Fan	Ae
Optimum temperature °C	46·0	46·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0	37·0
DNA G+C (mol%)	68·06 (T _m)	68·26 (T _m)	65·9 (T _m)	62 (HPLC)	NP	NP	NP	NP	NP	61 (T _m)	NP	50·9 (T _m)	70	NP	NP	50·9 (T _m)
Nitrate reduction	+	+	+	–	–	+	+	–	+	+	V	–	+	+	–	ND
Starch hydrolysis	+	+	ND	–	–	ND	–	+	ND	ND	ND	ND	–	+	–	ND
Aesculin hydrolysis	+	–	+	+	+	+	–	+	+	+	+	+	+	+	+	–
Substrates:																
D-Arabinose	+	+	–	–	–	V	+	–	–	+	–	–	–	–	–	–
Cellobiose	+	+	W	+	+	ND	ND	–	ND	ND	ND	ND	–	ND	–	–
Cyclodextrin	+	+		–	–	–	ND	ND	–	–	–	ND	ND	ND	ND	ND
Fructose	W	W	ND	+	+	ND	+	+	ND	ND	ND	ND	+	ND	+	ND
Glycogen	–	+	W	–	–	–	–	+	ND	–	–	–	–	+	–	ND
Inositol	+	–	–	ND	ND	ND	+	+	ND	ND	ND	–	+	+	–	–
Lactose	+	+	+	W	–	+	+	+	+	+	+	+	+	+	W	+
Mannitol	–	+	V	–	–	–	–	–	–	V	+	–	+	–	–	–
Mannose	+	–	–	+	ND	ND	+	–	ND	ND	ND	+	+	ND	+	+
Melibiose	+	+	ND	+	–	V	+	–	ND	+	+	+	+	ND	+	ND
Melezitose	+	+	W	–	–	–	–	–	+	+	+	V	+	ND	–	–
Raffinose	+	+	+	+	W(V)	+	+	–	+	+	+	+	+	+	+	+
Rhamnose	+	+	W	ND	ND	ND	–	–	ND	ND	ND	–	–	–	–	–
Ribose	+	+	+	–	–	+	+	–	+	V	ND	–	+	ND	–	–
Salicin	+	–	+	ND	+	ND	+	–	ND	ND	ND	–	+	+	–	W
Sorbitol	+	+	–	–	–	–	–	–	–	–	–	ND	–	ND	–	–
Trehalose	+	+	–	+	+	+	ND	–	+	+	+	+	+	+	–	+
D-Xylose	+	+	–	–	–	+	+	–	–	+	ND	+	+	–	–	–
Fermentation products (acids)	Formic, acetic, lactic	Formic, acetic, lactic	NP	Acetic, lactic	Acetic, lactic, succinic	Lactic, acetic, succinic	Formic, acetic, lactic, propionic	NP	Succinic, acetic, lactic	NP	NP	Acetic, lactic succinic	NP	NP	NP	Acetic, lactic succinic

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