Ruminococcus bovis sp. nov., a novel species of amylolytic Ruminococcus isolated from the rumen of a dairy cow

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Abstract
This study describes JE7A12T (=ATCC TSD-225T=NCTC 14479T), an isolate from the ruminal content of a dairy cow. Phenotypic and genotypic traits of the isolate were explored. JE7A12T was found to be a strictly anaerobic, catalase-negative, oxidase-negative, coccoid bacterium that grows in chains. The API 50 CH carbon source assay detected fermentation of d-glucose, d-fructose, d-galactose, glycogen and starch. HPLC showed acetate to be the major fermentation product as a result of carbohydrate fermentation. Phylogenetic analysis of JE7A12T based on 16S rRNA nucleotide sequence and amino acid sequences from the whole genome indicated a divergent lineage from the closest neighbours in the genus Ruminococcus. The results of 16S rRNA sequence comparison, whole genome average nucleotide identity (ANI) and DNA G+C content data indicate that JE7A12T represents a novel species which we propose the name Ruminococcus bovis with JE7A12T as the type strain.

The genus Ruminococcus was first described by Sijpesteijn [1] with Ruminococcus flavefaciens as the type strain [2]. Previously, members of the genus Ruminococcus have most frequently been isolated from the rumen and gastrointestinal tract of a wide variety of animals, including humans [3]. The genus is polyphyletic and divided into two groups. Ruminococcus group 1 includes the type strain Ruminococcus flavefaciens, Ruminococcus albus, Ruminococcus bromii and Ruminococcus callidus. Ruminococcus group 2 species have recently undergone taxonomic re-classification with many species being reassigned to different genera [4]. It is now believed that true members of the genus Ruminococcus are the species found in group 1 [4].

Microbial fermentation plays a prominent role in the utilization of feed by ruminants. In the rumen, bacterial fermentation is known to contribute to the stabilization of ruminal pH, increase volatile fatty acid production, reduce ammonia concentration and improve fibre digestibility [5–12]. Ruminococci are ubiquitous members of the human gastrointestinal and rumen microbial consortia worldwide where they play a role in the fermentation of cellulose rich feedstuffs and resistant starch [13–19]. Assessment of the global distribution of rumen microbes by Henderson et al. found that species of the genus Ruminococcus were present in all ruminants surveyed and, on average, were found to comprise 3.6% of the total rumen bacterial community [20]. While the abundance of members of the genus Ruminococcus is naturally high there is evidence that their functional role is larger than the abundance would suggest. Xia et al. revealed that 70–80% of the starch degrading bacteria in the barley-fed beef heifers were members of the family Ruminococcaceae [21]. Similarly, shotgun metagenomics approaches have demonstrated that a disproportionately high number of genes encoding hemicellulase and cellulase in the rumen can be associated with members of group 1 of the genus Ruminococcus [22]. Thus, characterization of novel species of the genus Ruminococcus has the potential to elucidate underlying microbial functionality in the rumen and the influence of members of the genus with regards to ruminant feed utilization and nutrition. The following description pertains to the isolation and classification of a novel group 1 amylolytic species, represented by strain JE7A12T, of the genus Ruminococcus.

ISOLATION AND ECOLOGY
JE7A12T was recovered from the rumen content of a healthy, Holstein dairy cow obtained from a farm in Tulare, California, USA on a modified chopped meat broth with carbohydrates solid medium (DSMZ Medium 110) at 37°C in an anaerobic
The medium was modified by the removal of fat-free ground meat and casein and the addition of 30.0 g peptone, 15.0 g meat extract, 10.0 g meat peptone, 15.0 g agar and 100 ml clarified rumen fluid per litre of medium. After 48 h of anaerobic incubation at 37–39 °C, JE7A12T displayed off-white colonies approximately 0.1–0.3 mm in diameter on supplemented Bacto Tryptic Soy Broth (BD) with 0.4 g l- cysteine hydrochloride, 0.02 g ferric ammonium citrate, 10 µg vitamin K1, 2.0 mg resazurin sodium salt, 10.0 ml vitamin supplement ATCC MDVS (ATCC) and 7.0 g Gelrite (CP Kelco) per litre of medium (TSB+FAC).

Gram-staining was performed as described by Beveridge [24]. Cell morphology was observed under an Accu-Scope EXC-350 light microscope using cells grown for 48 h at 37 °C on TSB+FAC. Cell size was measured using the microscopy imaging software Captavision + (Accu-Scope). Cells were Gram-stain positive, non-spore-forming and presented as small cocci (0.9–1.2 µm in diameter) (Figs S1 and S2, available in the online version of this article). The strain did not grow in the presence of oxygen and therefore is considered obligately anaerobic. Consistent with previous descriptions of the genus, JE7A12T is a strictly anaerobic coccoid, commonly found in pairs and chains [3]. Although isolated from rumen content, JE7A12T does not require rumen fluid for growth.

### 16S rRNA PHYLOGENY

16S rRNA based phylogeny was computed by the neighbor-joining method using mega X [25]. JE7A12T was placed in a dendrogram of all type strains of species from the order Clostridiales for which a full length 16S rRNA sequence was available in the RDP database [26]. The dendrogram was trimmed to include all the current members of the genus Ruminococcus as well as close phylogenetic neighbours (Fig. 1).

To confirm the results from the tree reconstructed from 16S rRNA sequences, a second phylogenetic tree was reconstructed using PhyloPhlan and a subset of 400 conserved proteins [27]. JE7A12T was placed in the dendrogram generated by PhyloPhlan with type strains of species of the genus Ruminococcus as well as type strains of species that were close matches from the 16S rRNA phylogenetic analysis (Fig. 2). Both the 16S rRNA tree reconstructed using mega X and
Table 1. At 34.6 mol%, the DNA G+C content of JE7A12\textsuperscript{T} resulted in the generation of a single, circular contig with a sequencing were generated using a Kapa HyperPlus kit of the genus. The lowest known DNA G+C content for other as it is significantly lower than those of any other member should act as a differentiating characteristic for the species JE7A12\textsuperscript{T} and all current members of the genus genome size and DNA G+C content were compared between deposited at NCBI (accession number CP039381). Whole of the assembly is 34.6 mol%. The whole genome has been resulted in greater than 100× coverage by Illumina reads the SQK- RAD004 kit (Oxford Nanopore Technologies) and in parallel, long-read libraries were generated using (Roche and single- end sequenced (1×300) on a MiSeq (Illu- nation protocol [28]. Short- read libraries for whole genome DNA from a pure culture of JE7A12\textsuperscript{T} was extracted by a modified Sambrook phenol–chloroform extraction/purification protocol [28]. Short- read libraries for whole genome sequencing were generated using a Kapa HyperPlus kit (Roche and single- end sequenced (1×300) on a MiSeq (Illu- mina). In parallel, long-read libraries were generated using the SQK- RAD004 kit (Oxford Nanopore Technologies) and 1D sequenced on the MinION (R9.4 flowcell). Sequencing resulted in greater than 100× coverage by Illumina reads and 55× coverage by Oxford Nanopore. The genome was assembled by hybrid methods, utilizing both Canu [29] and Pilon [30], as described by George et al. [31]. The assembly resulted in the generation of a single, circular contig with a length and N50 of 2440231 base pairs. The DNA G+C content of the assembly is 34.6 mol%. The whole genome has been deposited at NCBI (accession number CP039381). Whole genome size and DNA G+C content were compared between JE7A12\textsuperscript{T} and all current members of the genus Ruminococcus (Table 1). At 34.6 mol%, the DNA G+C content of JE7A12\textsuperscript{T} should act as a differentiating characteristic for the species as it is significantly lower than those of any other member of the genus. The lowest known DNA G+C content for other members of the genus is 39 mol% for strains of R. flavefaciens and R. bromii [3].

The full length 16S rRNA sequence of JE7A12\textsuperscript{T} was extracted from the whole genome sequence. The authenticity of the assembled 16S rRNA sequence was confirmed by comparison with a 16S rRNA amplicon sequence obtained using the 27F and 1492R primers and previously described methods [32]. The full length 16S rRNA sequence was subsequently compared with entries in the NCBI database by BLAST. Excluding species without validly published names, the closest neighbours to JE7A12\textsuperscript{T} based on 16S rRNA sequence similarity are Ruminococcus bromii (93.3%), Clostridium leptum (91.2%) and Caproiciproducens galactitolivorans (89.2%).

To further investigate taxonomic identity, whole genome average nucleotide identity (ANI) was compared between JE7A12\textsuperscript{T} and type strains for all current species of the genus Ruminococcus [33]. Additionally, type strains of Clostridium leptum and Caproiciproducens galactitolivorans were included in the ANI analysis due to their close 16S rRNA similarity. Due to bias in ANI algorithms, the ANI of JE7A12\textsuperscript{T} was evaluated utilizing both MUMmer and BLAST algorithms [34–36] (Tables 2 and 3). There were no matches at the suggested 95% cutoff for defining a species [33, 34, 37]. The best match by BLAST was to Ruminococcus bromii. However, the two species are genetically distant as their genomes share 72.7% sequence similarity but at only 20.1% coverage of the genome (Table 3). MUMmer offered higher sequence similarity matches than BLAST, with sequence alignment values between 81.7 and 93.9% for all species. However, these matches exhibited very low genome coverage (Table 2). The only species which demonstrates greater than 0.3% genome coverage was

**GENOME FEATURES**

DNA from a pure culture of JE7A12\textsuperscript{T} was extracted by a

**Fig. 2.** JE7A12\textsuperscript{T} phylogenetic tree by PhyloPhlan dendrogram; JE7A12\textsuperscript{T} and type strains of species of the genus Ruminococcus as well as type strains of other close phylogenetic neighbours. JE7A12\textsuperscript{T} is indicated in green type, type strains of members of Ruminococcus group 1 are indicated in blue type. Branch length based on relative concatenated amino acid sequence similarity is appended to each branch. NCBI GenBank accession numbers are appended to each species label.
Table 1. Characteristics of JE7A12^T compared with members of the genus Ruminococcus

Fermentation data for *R. champanellensis* and *R. gauveaeuii* were taken from Chassard et al. [15] and Domingo et al. [42], respectively. Fermentation data for all other species were taken from Ezaki [3]. +, Positive; −, negative; sd, strain dependent; w, weak reaction; nd., no data; A, acetate; F, formate; S, succinate; *, average based on all assemblies in the NCBI database.

| Characteristic | JE7A12^T | R. albus | R. bromii | R. callidus | R. champanellensis | R. flavifaciens | R. gauveaeuii | R. gnavus | R. lactaris | R. torques |
|----------------|---------|----------|-----------|-------------|-------------------|----------------|--------------|-----------|-------------|-----------|
| Genomic DNA G+C content (mol%) | 34.6 | 44.7* | 41.0* | 49.1* | 53.3* | 46.9* | 47.6* | 42.7* | 42.7* | 41.7 |
| Genome size (Mbp) | 2.44 | 3.71* | 2.15* | 3.10* | 2.54* | 2.70* | 4.10* | 3.50* | 2.73* | 3.00' |
| Major fermentation product(s) | A | A, F | A | A, S | A, S | A, F, S | A | A, F | A, F | A, L |
| Fermentation of: | | | | | | | | | | |
| Arabinose | – | – | – | – | – | ND | – | ND | + | – | – |
| Cellobiose | – | + | – | + | + | + | – | – | – | – |
| Glucose | + | + | + | + | – | – | ND | + | + | + |
| Lactose | – | + | – | + | – | + | – | – | + | + |
| Mannose | – | + | w/– | – | – | – | ND | – | w/- | w/- |
| Maltose | + | – | + | + | – | – | ND | + | sd | w |
| Mannitol | – | – | – | – | ND | – | ND | – | + | – |
| Raffinose | – | – | – | + | – | – | – | + | – | – |
were incubated at 37 °C for 72 h and monitored for growth. With pH tested in increments of 0.5 pH units. Hungate tubes to 2.5 %. Tolerance to pH (5.0–9.0) was tested on TSB+FAC determined by 16S rRNA sequence alignment.

| Genus species (Genbank accession number) | ANI (%) | Coverage (%) |
|------------------------------------------|---------|--------------|
| Ruminococcus gauvreauii CCRI-16110T (GCA_000425525) | 93.7 | 0.11 |
| Caproiciproducens galactitolivorans BS-1T (GCA_004768785) | 92.5 | 0.31 |
| Clostridium leptum VPI T7-24-1T (GCA_000154345) | 89.8 | 0.16 |
| Ruminococcus champanellensis 18P13T (GCA_0001210095) | 88.8 | 0.24 |
| Ruminococcus callidus VPI57-31T (GCA_000468015) | 87.8 | 0.20 |
| Ruminococcus flavefaciens ATCC 19208T (GCA_000518765) | 86.0 | 0.21 |
| Ruminococcus albus ATCC 27210T (GCA_000179635) | 85.8 | 0.14 |
| Ruminococcus bromii VPI 6883T (GCA_0002834225) | 84.5 | 1.66 |
| Ruminococcus granus VPI C7-9T (GCA_009831375) | 82.3 | 0.13 |
| Ruminococcus torques VPI B2-51T (GCA_000153925) | 82.2 | 0.18 |
| Ruminococcus lactaris VPI X6-29T (GCA_000155205) | 81.7 | 0.21 |

**Table 3. Average nucleotide identity by BLAST**

| Genus species (Genbank accession number) | ANI (%) | Coverage (%) |
|------------------------------------------|---------|--------------|
| Ruminococcus bromii VPI 6883T (GCA_002834225) | 72.8 | 20.2 |
| Ruminococcus torques VPI B2-51T (GCA_000153925) | 71.9 | 3.32 |
| Ruminococcus albus ATCC 27210T (GCA_000179635) | 71.6 | 2.76 |
| Ruminococcus flavefaciens ATCC 19208T (GCA_000518765) | 70.9 | 3.28 |
| Ruminococcus gauvreauii CCRI-16110T (GCA_000425525) | 69.3 | 1.38 |

**Ruminococcus bromii.** Whole genome nucleotide dissimilarity is a strong differentiator of JE7A12T from the other taxa of the genus.

**PHYSIOLOGY AND CHEMOTAXONOMY**

Catalase and oxidase activities of JE7A12T were determined using 3% (v/v) hydrogen peroxide solution and 1.2% tetra-methyl-p-phenylenediamine dihydrochloride solution, respectively. Growth temperature ranges were determined in TSB+FAC medium at 25, 30, 37, 40 and 50 °C. Optimal growth was observed at 37 and 40 °C, reduced growth at 30 °C and no growth at 25 and 50 °C incubation temperature. No motility was observed. Growth in the presence of salt was studied by supplementing TSB+FAC liquid medium with NaCl (0.5–4.5 % w/v in 0.5 % increments). Hungate tubes were incubated at 37 °C for 72 h and monitored for growth. JE7A12T was capable of growing in salt concentrations of up to 2.5 %. Tolerance to pH (5.0–9.0) was tested on TSB+FAC with pH tested in increments of 0.5 pH units. Hungate tubes were incubated at 37 °C for 72 h and monitored for growth. The optimal pH for growth was pH 7.0–7.5 with reduced growth at pH 6.0–6.5. No growth was observed at pH 8.0–9.0 and pH 5.0–5.5 on TSB+FAC.

Carbohydrate fermentation of JE7A12T was qualitatively measured using the API 50CH carbon panel (BioMérieux). JE7A12T cells were grown to late exponential phase and recovered by centrifugation at 3000 g for 10 min. Cells were resuspended and 0.017 % (w/v) bromocresol purple added as a pH indicator for acidification of carbohydrates [38]. JE7A12T fermented d-galactose, d-glucose, d-fructose, maltose, glycogen, aesculin/ferric citrate and starch. No fermentation of glycerol, erythritol, d-arabinose, l-arabinose, d-xylose, l-xylose, methyl β-d-xylopyranoside, d-cellobiose, d-adonitol, d-lactose, d-saccharose, d-trehalose, d-melibiose, d-mannose, l-arabitol, l-sorbose, l-rhamnose, dulcitol, inositol, d-mannitol, d-sorbitol, methyl d-mannopyranoside, methyl d-glucopyranoside, N-acetyl glucosamine, amygdalin, arbutin, melezitose, raffinose, xylitol, inulin, salicin, gentiobiose, turanose, d-lyxose, d-tagatose, d-fucose, l-trehalose, d-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate was observed (Table S1).

A comparison of carbon source fermentation between JE7A12T and all current species of the genus *Ruminococcus* can be found in Table 1. Similarly to *R. bromii*, JE7A12T shows narrow specialization with regards to carbohydrate fermentation, while other members of the genus *Ruminococcus* generally ferment a wider range of carbohydrates [39]. Specifically,
R. bromii has been reported to ferment most of the same carbon sources as JE7A12T, including galactose, glucose, fructose, maltose, glycogen and starch [3, 40]. Despite the similarities between the species, strains of R. bromii derived from the bovine rumen are not known to ferment fructose or galactose and are rarely able to ferment glucose. Utilization of these carbon sources have more commonly been observed in human derived R. bromii [41]. Therefore, fermentation of glucose, fructose and galactose could act to differentiate JE7A12T from ruminally derived Ruminococcus bromii.

Metabolite production was measured using a Waters Acquity UPLC Q System with RI detector. The column used was a Phenomenex 00H-0138-K0 Rezex ROA Organic Acid H+ (8%) operated at 60°C. The mobile phase was 0.001625 M H2SO4 at 0.5 ml min−1. Pure standards of acetate, ethanol, glycerol, lactate, butyrate, butanol, propionate, succinate and pyruvate were used for calibration at varying concentrations. JE7A12T produces acetate as a major fermentation product as well as ethanol and glycerol as minor products. No lactate, butyrate, butanol, propionate, succinate or pyruvate is produced. A comparison of metabolite production between JE7A12T and all current species of the genus Ruminococcus can be found in Table 1. The fermentation profile of JE7A12T most closely resembles that of R. bromii and R. gauvreauii which are the only species in the genus that produce acetate, and only acetate, as a major metabolic product. While the other members of the genus produce acetate, they also produce high levels of succinate, lactate and formate.

DESCRIPTION OF RUMINOCOCUS BOVIS SP. NOV.

Ruminococcus bovis (bo'vis. L. gen. n. bovis of the cow)

Ruminococcus bovis is an obligately anaerobic, catalase-negative and oxidase-negative bacterium. It is Gram-stain-positive and forms chains of small cocci when cultured in liquid medium. When cultured on TSB+FAC solid medium, it forms small, slightly opaque, off-white, circular colonies with even margins. Fermentation of D-galactose, D-glucose, D-fructose, maltose, glycogen, aesculin/ferric citrate and starch is indicated by API CH50. The major fermentation product is acetate, with ethanol and glyceral as minor products. No lactate, butyrate, butanol, propionate, succinate or pyruvate is produced.

The type strain is JE7A12T (=ATCC TSD-225T=NCTC 14479T) and was originally isolated from rumen content of a healthy, Holstein cow from Tulare, California, USA. The genomic DNA G+C content of the type strain is 34.6 mol%.

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Conflicts of interest
All authors are members of Native Microbials (formerly known as ASCUS Biosciences, Inc.) which provided funding for this project.

Ethical statement
Sampling procedures were approved by veterinarians at Dairy Experts (Tulare, CA) and Native Microbials, Inc.

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