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MICROBIOLOGY SOCIETY

Clostridium tanneri sp. nov., isolated from the faecal material of an alpaca

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Abstract

A strictly anaerobic, Gram-stain-negative rod-shaped bacterium, designated A1-XYC3^T, was isolated from the faeces of an alpaca (*Lama pacos*). On the basis of the results of a comparative 16S rRNA gene sequence analysis, the isolate was assigned to the genus *Clostridium* with the highest sequence similarities to *Clostridium magnum* DSM 2767^T (96.8%), *Clostridium carboxidivorans* P7^T (96.3%) and *Clostridium aciditolerans* JW/YJL-B3^T (96.1%). The average nucleotide identity between A1-XYC3^T, *C. magnum, C. carboxidivorans* and *C. aciditolerans* was 77.4, 76.1 and 76.6%, respectively. The predominant components of the cellular fatty acids of A1-XYC3^T were $C_{14:0}$, $C_{16:0}$ and summed feature 10, containing $C_{18:0}$/$C_{17:0}$ cyclo. The DNA G+C content was 32.4 mol%. On the basis of biochemical, phylogenetic, genotypic and chemotaxonomic criteria, this isolate represents a novel species within *Clostridium sensu stricto* for which the name *Clostridium tanneri* sp. nov. is proposed. The type strain of this species is strain A1-XYC3^T (=CCM 9376^T=NRRL B-65691^T).

INTRODUCTION

The genus *Clostridium* within the family *Clostridiaceae* historically encompassed Gram-stain-positive, rod-shaped, anaerobic and spore-forming bacteria [1]. However, some species of the genus *Clostridium* display Gram-stain negative or Gram-stain-variable reactions as stated in the current genus description [2]. Currently, there are more than 150 species of the genus *Clostridium* with validly published names according to the List of Prokaryotic Names with Standing in Nomenclature (LSPN;https://lpsn.dsmz.de/genus/clostridium, April 2024). The genus *Clostridium* is a genus with historically well-documented taxonomic issues [1–3], culminating in a genus displaying a wide range of observable phenotypes and metabolic properties. With the application of molecular methods and whole-genome sequencing, the sheer depth of phylogenetic diversity within organisms designated as members of the genus *Clostridium* began to be uncovered [2–4]. In 2016, Lawson and Rainey proposed restricting the genus *Clostridium* to *Clostridium butyricum* and its close relatives [2], now recognised as *Clostridium sensu stricto*. Members of *Clostridium sensu stricto* can be found throughout the collective mammalian gastrointestinal environment [5–8], as well as modified ruminants (sheep and alpaca). Upon examining the literature, it was evident that the majority of non-human mammalian microbiomes are understudied, and recent studies have revealed that other understudied mammalian species, such as bats [9] and pandas [10], have the potential to increase our understanding of the diversity of the mammalian gut microbiome. In a continuing study of mammalian microbiomes, using a polyphasic taxonomic approach, a novel species of the genus *Clostridium* belonging to *Clostridium sensu stricto*, designated as strain A1-XYC3^T, was recovered during the enrichment of faecal material from an alpaca (*Llama pacos*). Strain A1-XYC3^T represents a novel species of the genus *Clostridium* for which the name *Clostridium tanneri* sp. nov. is proposed.

ISOLATION AND ECOLOGY

Strain A1-XYC3^T was isolated from a freshly voided alpaca faecal sample obtained from a domesticated alpaca on a privately owned alpaca ranch near Newcastle, Oklahoma, USA (35.24 N, 97.59 W). The freshly voided faecal sample was collected, processed and transported anoxically on ice to the Lawson Microbial Systematics Laboratory at the University of Oklahoma, Norman, for further processing.

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Keywords: 16S rRNA; *Clostridium tanneri*; genome; *in-silico*; novel species; phylogeny; taxonomy.
Abbreviations: ANI, average nucleotide identity; BV-BRC, Bacterial and Viral Bioinformatics Resource Center; dDDH, digital DNA-DNA hybridisation; orthoANIu, orthologous ANI using USEARCH.
The 16S rRNA gene and genome sequences of strain A1-XYC3^T have been deposited in GenBank under the accession numbers OQ724631 and GCA_033659955.1, respectively.
Three supplementary figures and one supplementary table are available with the online version of this article.

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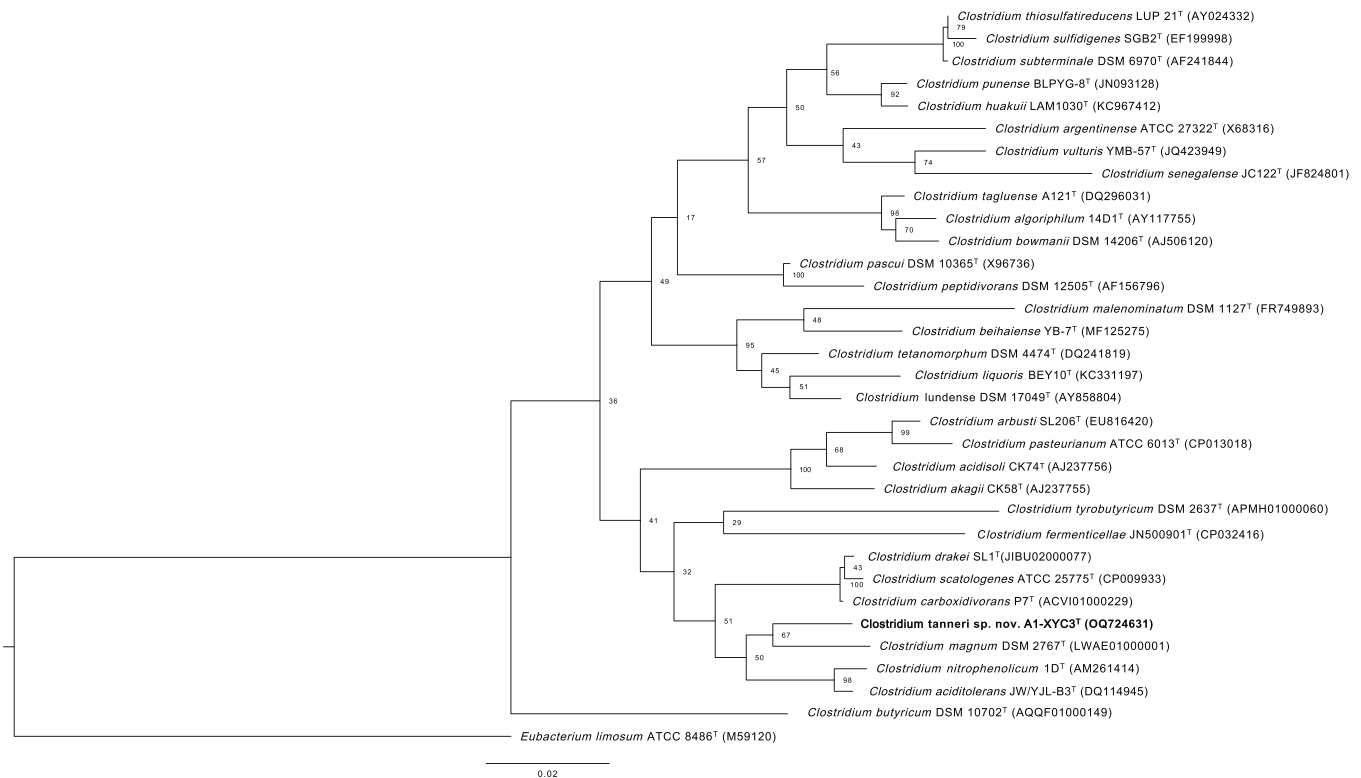

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationships between *Clostridium tanneri* sp. nov. A1-XYC3^{T} within *Clostridium sensu stricto*. Bootstrap values (percentages) were obtained with 1000 replicates and are displayed at their branch nodes. *Eubacterium limosum* ATCC 8486^{T} (M59120) was used as the outgroup. Bar, 0.02 substitutions per site.

Multiple anoxic enrichments, using xylan, pectin and cellulose as sole substrates [all added to 2% (w/v)], were prepared using 1.0 ml faecal slurry in a sealed serum bottle containing 100 ml modified Medium 2 (per 100 ml distilled water): casitone (1.0 g), yeast extract (0.25 g), minerals solution (A) (15.0 ml), minerals solution (B) (15.0 ml), clarified rumen fluid (20 ml), resazurin (0.0001 g), sodium lactate (70% w/v) (1.0 g), xylan (0.2 g), cysteine HCl (0.05 g), sodium bicarbonate (0.4 g) and distilled water (to 1000 ml). Minerals solution (A) contained (per 1000 ml); K_2HPO_4 (3.0 g). Minerals solution (B) contained (per 1000 ml); KH_2PO_4 (3.0 g), $(NH_4)_2SO_4$ (6.0 g), NaCl (6.0 g), $MgSO_4 \times 7H_2O$ (0.6 g) and $CaCl_2$ (0.6 g) as described previously [11]. The enrichment was incubated at 37°C for 14 days with a headspace of 5% hydrogen, 10% carbon dioxide and 85% nitrogen to maintain anoxic conditions. After incubation, samples were inoculated onto Medium 2 Agar containing the same sole carbon source as their original enrichment. Isolates were then sub-cultured onto BD Bacto Brain–Heart Infusion (BHI; BD) agar plates supplemented with 5% defibrinated sheep's blood via multiple plate streaks until pure isolates were obtained, as determined by Gram-stain-reaction and phase contrast microscopy (CX41, Olympus). One of the resulting isolates was designated A1-XYC3^{T}. A1-XYC3^{T} was isolated from an enrichment of modified Medium 2 supplemented with xylan. After primary isolation, A1-XYC3^{T} was maintained under anoxic conditions on BHI plus 5% sheep's blood medium at 37°C and preserved in BHI plus 5% clarified rumen fluid with 20% (v/v) glycerol at −80°C.

16s rRNA gene phylogeny

A1-XYC3^{T} was sent to GENEWIZ (Genewiz.com, South Plainfield, NJ, USA), where a near full-length 16S rRNA gene sequence (1399 bps) was obtained using Sanger sequencing. The nearest phylogenetic neighbours of A1-XYC3^{T} were determined by a BLAST search against the GenBank database and EzBioCloud's Identify [12–14]. The 16S rRNA gene sequences of A1-XYC3^{T}, its nearest neighbours, in addition to the type strain of the type species of the genus *Clostridium*, *Clostridium butyricum* DSM 10702^{T} (AQQF01000149), were downloaded from EzBioCloud, and these sequences were aligned using MUSCLE [15] and trimmed in MEGA 11 version 11.0.13 [16]. *Eubacterium limosum* ATCC 8486^{T} (M59120) was used as an outgroup. Phylogenetic trees were reconstructed according to the maximum-likelihood method [17] (Fig. 1) and neighbor-joining method [18] (Fig. S1, available in the online version of this article) using MEGA 11 software and the the Tamura–Nei substitution model [19] and bootstrap analyses were performed using 1000 replications. The results of phylogenetic analyses based on 16S rRNA gene sequences indicated that A1-XYC3^{T} was most closely related to *Clostridium magnum* DSM 2767^{T} (96.8%), *Clostridium carboxidivorans* P7^{T} (96.3%) and *Clostridium aciditolerans* JW/YJL-B3^{T} (96.1%). These 16S rRNA gene similarity values, along with the phylogenetic trees reconstructed using the maximum-likelihood method [17] (Fig. 1) and the neighbor-joining

method [18] (Fig. S1) support the proposal that A1-XYC3^T represents a novel species. Additionally, the 16S rRNA gene sequence similarity between A1-XYC3^T and its close relatives is below the currently accepted threshold of 98.8%, which is now routinely used to demarcate this taxonomic rank [20].

GENOME FEATURES

Cell biomass for A1-XYC3^T was gathered from an overnight culture, and genomic DNA was extracted using the DNeasy DNA extraction kit (Qiagen) following the manufacturer's protocol. The DNA yield was quantified using a Qubit 3.0 fluorometer, and 100 ng of DNA was used as input for genomic library construction. Briefly, the DNA was sheared using a sonicator (800R, QSonica), followed by bead cleanup to remove short fragments (< 150 bp). Next, PCR sequencing libraries were generated using Illumina-specific barcoded primers and pooled from triplicate reactions. Finally, sequencing libraries were purified using bead cleanup (1.5×), quantified using a Tape Station, pooled in equimolar proportions and sequenced on a MiSeq (Illumina).

Using SPAdes v3.13.0 [21], the genome for A1-XYC3^T was assembled and annotated using the Bacterial and Viral Bioinformatics Resource Centre [22] (BV-BRC) (https://www.bv-brc.org/). In addition, genomic sequences of close relatives of A1-XYC3^T and the type strain of the type species of *Clostridium sensu stricto*, *Clostridium butyricum* DSM 10702^T (GCA_932751065.1), were downloaded from BV-BRC.

The evolutionary relationships between A1-XYC3^T (GCA_033659955.1) and its nearest neighbours were evaluated using a phylogenetic approach. A whole-genome-based phylogenetic tree was reconstructed using the CodonTree method within the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) [22] and rapid randomised accelerated maximum likelihood (RAxML) analysis v8.2.11 [23, 24]. The support values for the phylogenetic tree were generated using 100 rounds of the 'rapid bootstrapping' option of RaxML, and visualisation was accomplished with the Interactive Tree Of Life v3 [25]. *Eubacterium limosum* ATCC 8486^T (GCA_000807675.2) was used as an outgroup. The taxonomic status of A1-XYC3^T was confirmed by comparing average nucleotide identity (ANI) and digital DNA–DNA hybridisation (dDDH) values between A1-XYC3^T and its close relatives. Values for ANI between A1-XYC3^T and close relatives were calculated using EzBioCloud's ANI Calculator, which uses the orthologous ANI using USEARCH (OrthoANIu) algorithm [13, 26]. The Genome-to-Genome Distance Calculator [27, 28] v3.0 was used to determine digital DNA–DNA hybridisation. Prodigal [29]was used to determine the DNA G+C (mol %) content.

Once assembled, the draft genome sequence of A1-XYX3^T consisted of 91 contigs, with a genome length of 3.9 Mbp, an N50 length of 150 735 bp and an average DNA G+C content of 32.4 mol%. Annotation in BV-BRC revealed 3857 protein-coding sequences, 65 tRNA genes and 8 rRNA genes. The OrthoANIu values between A1-XYX3^T and close relatives were 75.6–77.4%. Values for dDDH between A1-XYX3^T and the six nearest neighbours were less than 23.5% (Table 1). These ANI and dDDH values indicated that A1-XYX3^T represents a novel species within the genus *Clostridium sensu stricto*, as ANI and dDDH values lower than 95–96% and 70%, respectively, are established as the boundary for defining a prokaryotic species [27, 30]. The phylogenetic tree (Fig. 2) was consistent with the phylogenetic placement of A1-XYX3^T from the 16S rRNA gene tree (Fig. 1). The main genotypic characteristics of A1-XYC3^T and close relatives are given in Table 1.

In this study, an *in-silico* approach was used to investigate glycerophospholipid metabolism, the production of polar lipids was performed using the Kyoto Encyclopaedia of Gene and Genomes (KEGG) database [31]. An *in-silico* approach is now encouraged

Table 1. Genotypic differential characteristics ofA1-XYC3^T and close relatives

Strains: 1, A1-XYC3^T; 2, *Clostridium drakei* SL1^T; 3, *Clostridium scatologenes* ATCC 25775^T; 4, *Clostridium carboxidivorans* P7^T; 5, *Clostridium magnum* DSM 2767^T; 6, *Clostridium nitrophenolicum* 1D^T; 7, *Clostridium aciditolerans* JW/YJL-B3^T, NR, Not reported.

Characteristic	1	2	3	4	5	6	7
DNA G+C content (mol%)	32.4	29.7	29.6	29.9	32.1	35.5	30.8
16S sequence accession number	OQ724631	JIBU02000077	CP009933	ACVI01000229	LWAE01000001	AM261414	DQ114945
16S sequence similarity (%)	100.0	96.5	96.7	97.0	96.4	96.8	96.1
Genome accession number	GCA_033659955.1	GCA_003096175.1	GCA_000968375.1	GCA_001038625.1	GCA_900129955.1	NR	GCA_016316925.1
OrthoANIu (%)	100.0	75.8	75.6	76.1	77.4	NR	76.6
dDDH (%)	100.0	21.9	21.8	21.7	23.5	NR	23.1

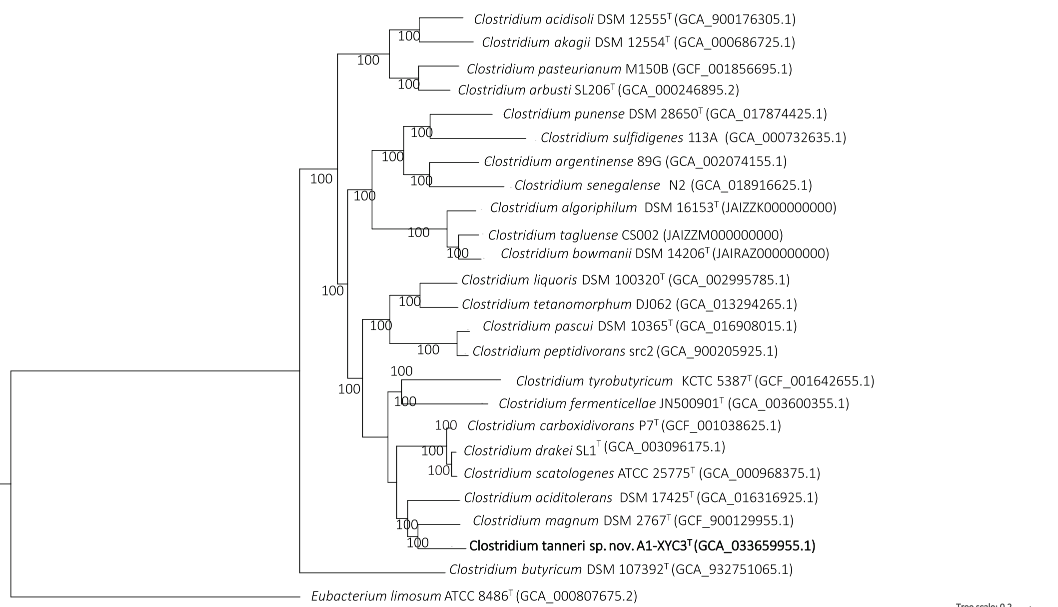

Fig. 2. Maximum-likelihood phylogenetic tree based on 605 single-copy genes, showing the relationships between *Clostridium tanneri* sp. nov. A1-XYC3^T and members of the genus *Clostridium sensu stricto*. A total of 610587 aligned nucleotides and 203529 amino acids to reconstruct this tree. Bootstraps were generated with 100 rounds of the 'Rapid' bootstrapping option within RaxML. *Eubacterium limosum* ATCC 8486^T (GCA_000807675.2) was used as the outgroup. Bar indicates the mean number of substitutions per site, 0.2.

and routinely utilised for polar lipid profiling [32–36], and along with biochemical traits, these methods are now routinely used for species descriptions [37–39]. The results (Fig. S2) indicate that A1-XYC3^T contains the enzymes phosphatidylglycerophate synthetase (E.C. 2.7.8.5) and phosphatidylglycerophate phosphatase (E.C. 3.1.3.27) required to synthesise phosphatidylglycerol (PG) from the essential building block cytidine diphosphate diacylglycerol. In addition, the enzyme cardiolipin synthase (E.C. 2.7.8.41) is present; this enzyme is required to produce diphosphatidylglycerol (DPG; cardiolipin). Furthermore, the genome of A1-XYC3^T also contains phosphatidylserine synthase (E.C. 2.7.8.8) used in the synthesis of phosphatidyl-L-serine (PS) an intermediate compound that the enzyme phosphatidylserine decarboxylase (PSD) (E.C. 4.1.1.65) further catalyses in the production of phosphatidylethanolamine (PE) from phosphatidyl-L-serine (Fig. S2). The identification of PS is interesting as this phospholipid is commonly absent from chromatography plates due to the conversion efficiency of PSD. Therefore, on the basis of the results of this analysis, the major polar lipids predicted to be produced by A1-XYC3^T are PE, PG, PS and DPG, consistent with those of other closely related species (Fig. S2) and other members of the genus *Clostridium sensu stricto* [40] determined using *in-silico* [36, 41] and wet lab methodologies [42]. The results of additional *in-silico* analysis of the cell wall of A1-XYC3^T indicated the presence of UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase (MurE) (E.C. 6.3.2.13), which is used to insert diaminopimelic acid (DAP) into the stem peptide of the peptidoglycan (Fig. S3) [43]. The presence of the diagnostic diamino acid DAP is consistent with the phenotypes of other members of the genus *Clostridium sensus stricto* [1, 2].

PHYSIOLOGY AND CHEMOTAXONOMY

The Gram stain reaction was determined using the Advanced Gram Stain Kit (Hardy Diagnostics) following the manufacturer's instructions. Aerotolerance was determined by placing cultures on media exposed to air. Cells were inspected for motility and endospore production by phase contrast microscopy (CX41, Olympus) at 1000× magnification.

Temperature ranges for growth were determined in BHI broth (supplemented with 5% (v/v) clarified rumen fluid) at 4, 20, 37, 45, 50 and 55°C. Salt tolerance was determined in BHI [supplemented with 5% (v/v) clarified rumen fluid] and tested at 0.5% (w/v) NaCl and between 1.0% (w/v) and 10.0% (w/v), in increments of 1.0% (w/v); pH ranges for growth were determined between 5.0 and 9.0, in increments of 1.0, and all tests were performed in duplicate.

For determination of the pH ranges, the following buffers were prepared at a final concentration of 10 mM; sodium acetate (pH 5.0), potassium phosphate (pH 6.0–8.0) and Tris-HCl (pH 9.0). Optimal growth conditions were determined from OD_{600} using a spectrophotometer (Spectronic 20D+, Milton Roy). An increase in OD_{600} greater than 0.1 after 5 days of incubation was considered growth. Additional biochemical traits were determined using the API ZYM test systems (bioMérieux) according to the manufacturer's instructions and performed in duplicate. Additionally, end products of metabolism for A1-XYC3^T were measured

Table 2. Morphological and physiological differential characteristics of A1-XYC3ᵀ and close relatives

Strains: 1, A1-XYC3ᵀ (data from this study); 2, *Clostridium drakei* SL1ᵀ [47, 48]; 3, *Clostridium scatologenes* ATCC 25775ᵀ [47, 48]; 4, *Clostridium carboxidivorans* P7ᵀ [47]; 5, *Clostridium magnum* DSM 2767ᵀ [49]; 6, *Clostridium nitrophenolicum* 1Dᵀ [42]; 7, *Clostridium aciditolerans* JW/YJL-B3ᵀ [50]. NR, Not reported.

Characteristic	1	2	3	4	5	6	7
Gram-stain reaction	−	−	−	+	−	+	−
Endospore production	−	+	+	+	+	+	+
Motility	−	+	+	+	+	+	+
Temperature range	20–45°C	18–42°C	18–42°C	24–42°C	15–45°C	20–45°C	20–45°C
Temperature optimum	37°C	30–37°C	37–40°C	37–40°C	30–32°C	30°C	35.5°C
pH range	6.0–7.0	4.6–7.8	4.6–8.0	4.4–7.6	6.0–7.5	6.5–8.0	3.8–8.9
pH optimum	7.0	5.4–7.5	5.4–7.0	5.0–7.0	7.0	7.2	7.0–7.5
Salinity range (%)	0.0–2.0	NR	NR	NR	NR	0.0–1.0	0.0–1.5
Optimum salinity (%)	0.5	NR	NR	NR	NR	NR	0.0
Source	Alpaca faeces	Acidic mine sediment	Acidic mine pond	Settling lagoon	Enriched sediment	Subsurface soil	Wetland sediment

from cultures grown in basal medium with glucose at 37°C. Analysis was performed in duplicate from duplicate cultures using GC (Agilent 6890 N GC, Agilent Technologies) as described by Liu *et al.* [44].

The cells of A1-XYC3ᵀ are Gram-stain-negative rods (Table 2). A1-XYC3ᵀ was strictly anaerobic. Cultures did not grow when the media was exposed to air. Colonies on BHI with 5% sheep's blood medium were opaque and circular on the agar surface after 24 h at 37°C. Cells were negative for motility and endospore production, visible with phase-contrast microscopy. A1-XYC3ᵀ could grow at between 20°C and 45°C with optimum growth at 37°C. A1-XYC3ᵀ could grow at NaCl concentrations between 0.5 and 2.0% (w/v) with optimal growth occurring at 0.5% (w/v) NaCl. A1-XYC3ᵀ grew at pH values between pH 6.0 to 8.0 with an optimum pH of 7.0. A1-XYC3ᵀ produced ethanol and acetate from glucose fermentation. The API ZYM test system detected positive reactions for esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative reactions were obtained for alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cysetine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Chemotaxonomic features were determined at the Centre for Microbial Identification and Taxonomy (University of Oklahoma, Norman, Oklahoma). The cellular fatty acids of A1-XYC3ᵀ were harvested, extracted and analysed using the Microbial Identification System (MIDI) [45, 46]. Analysis was performed with a 6890 N gas chromatograph (Agilent Technologies) equipped with a phenyl methyl silicone fused silica capillary column (HP-Ultra 2.25m×0.2 mm×0.33 µm film thickness) coupled with a flame ionisation detector. Hydrogen was used as the carrier gas. The temperature programme was preset at 170°C and increased at 5°C min^{-1} to a final temperature of 270°C. Fatty acids were identified and expressed in percentages using the QBA1 peak naming database within the MIDI system.

Major components of the fatty acids profile (≥10.0%) were $C_{14:0}$, $C_{16:0}$ and summed feature 10 (consisting of $C_{18:0}$/$C_{17:0}$ cyclo). Additionally, summed features 3, 4 and 12 represented the fatty acids present in moderate amounts (5.0%–9.9%) (Table S1), available in the online version of this article, which is consistent with the phenotypes of the members of *Clostridium sensu stricto*.

On the basis of biochemical, phylogenetic, genotypic and chemotaxonomic criteria, A1-XYC3ᵀ represents a novel species within the genus *Clostridium sensu stricto*. In addition to the phylogenetic trees (Figs 1 and 2 and S1), phenotypic and chemotaxonomic traits indicate the separateness of strain A1-XYC3ᵀ from its close relatives (Tables 1 and 2). It is proposed to name the novel strain A1-XYC3ᵀ as *Clostridum tanneri* sp. nov.

DESCRIPTION OF CLOSTRIDIUM TANNERI SP. NOV.

Clostridium tanneri (tan'ne.ri. N.L. gen. n. tanneri, of Tanner, in honour of Ralph S. Tanner, a contemporary American microbiologist for his many contributions to the field of anaerobic microbial cultivation).

The cells are strictly anaerobic, motility-negative, Gram-stain-negative rods in which sporulation was not observed. Optimal growth was observed at 37°C, with 0.5% (w/v) NaCl, at pH 7. Positive for growth at 25, 30, 37 40 and 45°C and in 1 and 3%, but not 10% (w/v) NaCl. Using the API ZYM test system positive reactions are observed for esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Ethanol and acetate were produced as fermentative end products. Negative reactions were obtained for alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The major components of the fatty acids profile are $C_{14:0}$, $C_{16:0}$ and summed feature 10 (containing $C_{18:0}/C_{17:0}$ cyclo).

The type strain is A1-XYC3^{T} (=CCM 9376^{T}=NRRL B-65691^{T}), which was isolated from the faeces of a domesticated alpaca in Newcastle, Oklahoma. The DNA G+C content of the type strain is 32.4 mol%. The 16S rRNA gene and genome sequences of A1-XYC3^{T} have been deposited in GenBank under the accession numbers OQ724631 and GCA_033659955.1, respectively.

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Conflicts of interest
The authors declare that they have no indirect or direct conflicts of interest.

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