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TAXONOMIC DESCRIPTION

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Clostridium bovifaecis sp. nov., a novel acetogenic bacterium isolated from cow manure

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Abstract

A strictly anaerobic, Gram-staining-positive, spore-forming rod-shaped bacterium, and designated BXX^T, was isolated from cow manure. Colonies on DSMZ medium 311c agar plates were cream, circular, opaque and lustrous. Growth occurred at 20–45 °C with a pH range of 5.0–10.0 and at NaCl concentrations of up to 2 % (w/v). The optimum temperature, pH and NaCl concentration for growth were 30 °C, pH 7 and 1 % (w/v), respectively. The major cellular fatty acids were $C_{16:0}$ (26.8 %), $C_{14:0}$ (22.8 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) (16.4 %) and $C_{16:1}\omega9c$ (10.7 %). The main polar lipids of BXX^T were diphosphatidylglycerol, phosphatidylethanolamine, unidentified aminolipids, an unidentified phospholipid and unidentified lipids. Acetate was mainly produced from H_2/CO_2, $H_2/CO_2/CO$ (4/3/3, v/v/v), formate, glycerol, 1,2-propanediol, pyruvate, D-fructose and 2-methoxyethanol. BXX^T is most closely related to *Clostridium thermobutyricum* DSM 4928^T, *Clostridium homopropionicum* DSM 5847^T and *Clostridium thermopalmarium* DSM 5974^T with 16S rRNA gene sequence similarities of 96.9, 96.6 and 96.5 %, respectively. The DNA G+C content of BXX^T was 33.7 mol%, which was lower than that of *C. thermobutyricum* DSM 4928^T (37.0 mol%) and *C. thermopalmarium* DSM 5974^T (35.7 mol%). In addition, DSM 4928^T and DSM 5974^{T}are thermophilic members of the genus *Clostridium*. The absence of $C_{15:0}$ also distinguished BXX^T from *Clostridium thermobutyricum*. On the basis of phylogenetic, phenotypic and chemotaxonomic evidence, the novel isolate represents a novel species within the genus *Clostridium*, for which the name *Clostridium bovifaecis* sp. nov is proposed. The type strain of the type species is BXX^T (=JCM 32382^T=CGMCC 1.5228^T).

Acetogenic bacteria are anaerobes that use the acetyl-coenzyme A (CoA) Wood–Ljungdahl pathway for the reduction of CO_2 to acetyl-CoA, the conservation of energy, and the assimilation of CO_2 into cell carbon [1, 2]. Acetogenic bacteria are a key component of the global anaerobic food web, which produce 10 % of the world's total annual output of acetate and play an important part in the global carbon cycle [3]. Furthermore, the metabolic flexibility of acetogens gives these bacteria an ecological advantage [4]. The number of known acetogens has increased significantly in the last two decades although it is pertinent to note that acetogenesis is not a phylogenetic trait. Over 100 different species have been isolated, and they have been assigned to 22 different genera [5]. Here we report a new acetogen, strain BXX^T, obtained from cow manure. On the basis of the phylogenetic and phenotypic features of this strain, we propose a novel species of the genus *Clostridium*.

BXX^T was isolated using the Hungate technique [6]. Pure cultures were grown in DSMZ medium 311c. DSMZ medium 311c (per litre) has the following composition: 0.50 g NH_4Cl, 0.50 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2.2H_2O$, 2.25 g NaCl, 2.00 mg $FeSO_4.7H_2O$, 1.00 ml trace element solution SL-10 (DSMZ medium 320), 1.00 ml selenite–tungstate solution (see DSMZ medium 385), 2.00 g yeast extract, 2.00 g casitone, 0.50 ml Na–resazurin solution (0.1 % w/v), 0.35 g K_2HPO_4, 0.23 g KH_2PO_4, 4.00 g $NaHCO_3$, 10.00 ml vitamin solution (see DSMZ medium 141), 0.30 g L-cysteine-HCl.H_2O, 0.30 g $Na_2S.9H_2O$, distilled water added to 1000 ml final volume. The pH was adjusted to 7.0. Colonies appeared after incubation on DSMZ agar medium 311c for 3 days. The strain was picked as a single colony and purified by transferring it to fresh DSMZ agar medium 311c at least three times. The strain was stored in 25 % glycerol (v/v) solution at −80 °C for further study. Pure cultures

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Abbreviations: CGMCC, China General Microbiological Culture Collection Center; DDBJ, DNA Data Bank of Japan; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; EMBL, European Bioinformatics Institute; JCM, Japan collection of microorganisms.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BXX^T is MH346511. The GenBank/EMBL/DDBJ accession number for the genome of strain BXX^T is SRP145535.

One supplementary figure is available with the online version of this article.

grown in DSMZ liquid medium were transferred into the medium 311c in anaerobic bottles (250 ml), then filled with a gas mixture of H_2/CO_2 (4 : 1).

For morphological, physiological and biochemical characterization, BXX^T was cultivated on DSMZ medium 311c at pH 7.0 and 30 °C for 3 days. Cell morphology was examined under a digital biological microscope (CX31RTSF, Olympus) and transmission electron microscope (H-7650, Hitachi). Gram staining was carried out according to the methods of Johnson *et al.* [7].

Colonies on DSMZ agar medium 311c were 0.5–3 mm in diameter after 5 days of growth. Colonies were cream, circular, opaque and lustrous. Colonies turned from cream to brown after 2 weeks of growth. No growth was observed when grown aerobically, so BXX^T is anaerobic. Cells of BXX^T were Gram-staining-positive, motile and rod-shaped with a cell size of 0.20 to 0.57 µm in width and 1.60 to 6.10 µm in length. Endospores formed when cells lacked sufficient nutrition or the metabolic concentration was too high. Cells always appeared as single cells or longer chains (See Fig. S1, available in the online version of this article). The morphology of BXX^T is consistent with those of members of the genus *Clostridium* [8].

Genomic DNA was extracted and purified by using a MoBio Powersoil DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's instructions. The 16S rRNA gene sequence was amplified using the method described by Hutson *et al.* [9]. A BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the 16S rRNA gene sequence against the GenBank database detected closely related sequences that were retrieved for phylogenetic analysis. The phylogenetic tree was reconstructed by the neighbor-joining method with MEGA 6.06 software based on a Geneious 7.1.7 CLUSTAL W alignment [10, 11].

The 16S rRNA gene sequence of BXX^T was 1502 bp long. Phylogenetic analysis based on sequencing of the 16S rRNA gene indicated that BXX^T represented a member of the genus *Clostridium*. The maximum-likelihood phylogenetic tree showed the phylogenetic relationships between BXX^T and other members of the genus *Clostridium* (Fig. 1). The phylogenetically closest strains to BXX^T were *Clostridium thermobutyricum* DSM 4928^T, *Clostridium homopropionicum* DSM 5847^T and *Clostridium thermopalmarium* DSM 5974^T, with 96.9, 96.6 and 96.5 % similarity, respectively. BXX^T showed less than 95.0 % 16S rRNA gene similarity with the type species of the genus *Clostridium*, *Clostridium butyricum*, and other related species of the genus *Clostridium*, but it falls within *Clostridium sensu stricto* [8].

For the determination of temperature and pH range, cells were grown in DSMZ medium 311c at 10, 15, 20, 25, 30, 35, 37, 40, 45, 50 and 55 °C and pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. The pH was adjusted with NaOH or HCl solutions [12]. To determine the effect of NaCl on the growth of BXX^T, NaCl was weighed directly into the medium to give final concentrations of between 0 and 3 % (w/v) [13]. Growth at different pH values, NaCl concentrations and temperatures was detected after 3 days when these strains were in the exponential growth phase. CO, CO_2 and H_2 was measured by gas chromatography (FuLi) equipped with a stainless-steel column (AE. TDX-01, 2 m×3 mm). VITEK 2 Compact was used to test the utilization of various substrates as sole carbon and energy sources, according to the manufacturer's instructions.

Characteristics of BXX^T and comparisons with *Clostridium thermobutyricum* DSM 4928^T [14], *Clostridium homopropionicum* DSM 5847^T [15] and *Clostridium thermopalmarium* DSM 5974^T [16] are shown in Table 1. BXX^T grew at temperatures ranging from 20 to 45 °C with an optimum of 30 °C, while *Clostridium thermobutyricum* DSM 4928^T [14] and *Clostridium thermopalmarium* DSM 5974^T [16] are thermophilic members of the genus *Clostridium*. The pH range for growth of BXX^T was 5.0–10.0 with a pH optimum of 7.0. The strain grew under NaCl concentrations ranging from 0 to 2.0 % (w/v), and no growth was observed with 3.0 % (w/v) NaCl or higher, while growth of DSM 5847^T was totally inhibited at 1.0 % NaCl. BXX^T can grow with casein while DSM 4928^T and DSM 5974^T cannot. Yeast extract was required for

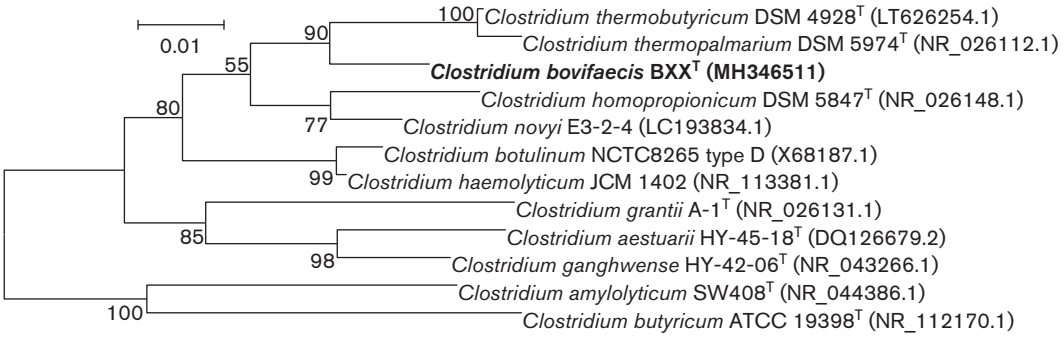

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1502 bp) showing the phylogenetic position of BXX^T, its closest relatives from the genus *Clostridium* and other related species. The tree was reconstructed with bootstrap values based on 1000 replications; only values >50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 evolutionary distances.

Table 1. Characteristics of BXX^T in comparison with type strains of the phylogenetically most closely related species of the genus *Clostridium*

Strains: 1, BXX^T; 2, *C. thermobutyricum* DSM 4928^T [14]; 3, *C. homopropionicum* DSM 5847^T [15]; 4, *C. thermopalmarium* DSM 5974^T [16]. +, Positive or good growth; –, negative or no growth; ND, not detected.

Characteristic	1	2	3	4
Source	Cow manure	Horse manure	Anoxic digester sludge	Palm wine
Cell width (μm)	0.20–0.57	0.90–1.10	1.20–1.50	0.70–1.00
Cell length (μm)	1.60–6.10	2.00–4.50	5.50–10.00	2.00–8.00
Growth temperature (°C)				
Range	20–45	26–61.5	20–40	ND
Optimum	30	55	37	55–60
Growth pH				
Range	5.0–10.0	5.8–9.0	5.6–8.3	6.0–8.2
Optimum	7.0	6.8–7.1	7.2	6.6
DNA G+C content (mol%)	33.7	37.0	32.0±1.0	35.7±0.3
Utilization of:				
H_2+CO_2	+	–	–	–
Formate	+	–	ND	ND
D-mannose	+	–	ND	ND
β-galactose	+	–	ND	–
α-galactose	+	–	ND	–
Arginine	–	+	ND	ND
D-ribose	–	+	ND	+
D-xylose	–	+	–	+
Glycerol	+	ND	–	–
Growth substrates:				
Yeast extract	+	+	+	+
Casein	+	–	ND	–

growth of DSM 4928^T and of DSM 5974^T. BXX^T produced acetate from H_2/CO_2(H_2 : CO_2=4 : 1), syngas (H2 : CO_2 : CO=4 : 3 : 3), formate, glycerol, 1,2-propanediol, pyruvate, D-fructose and 2-methoxyethanol, but *C. thermobutyricum* DSM 4928^T, *C.homopropionicum* DSM 5847^T and *C. thermopalmarium* DSM 5974^T cannot autotrophically grow with H_2/CO_2, thus BXX^T was shown to be acetogenic. According to the results obtained in VITEK 2 Compact tests, all of the following reactions are positive: cellobiose, D-glucose, D-mannose, maltose, sucrose, β-galactose, α-galactose, β-mannitol, maltotriose. All of the following reactions are negative: D-galactose, leucine, phenylalanine, L-proline, L-pyrrolydonyl, tyrosine, alanine–phenylalanine–proline, *N*-acetyl-D-glucosamine, β-glucose, urea, α-arabinose, arginine, β-D-fucose, phosphate, L-arabinose, D-ribose, phosphates, 5-bromo-4-chloro-3-hydroxyindoline-B-*N*-acetylglucosamine, α-L-arabinose, D-xylose, 5-bromo-4-chloro-3-indole-α-D-mannoside, α-L-fucose. D-ribose and D-xylose can be used by *C. thermobutyricum* DSM 4928^T and *C. thermopalmarium* DSM 5974^T. In addition, *C. thermobutyricum* DSM 4928^T was not able to utilize formate, D-mannose, β-galactose and α-galactose; *C. homopropionicum* DSM 5847^T was not able to utilize D-xylose and glycerol and *Clostridium thermopalmarium* DSM 5974^T was not able to utilize β-galactose, α-galactose and glycerol. Therefore, BXX^T differs from its closest phylogenetic neighbours (*C. thermobutyricum* DSM 4928^T, *C. homopropionicum* DSM 5847^T and *C. thermopalmarium* DSM 5974^T).

For fatty acid analyses, cell biomass was collected after 3 days of cultivation in DSMZ medium 311c at 30 °C. Identification was according to the standard protocol provided by the manufacturer (Sherlock Microbial Identification System; MIDI). For the determination of polar lipids, BXX^T was cultivated on DSMZ medium 311c at pH 7.0 and 30 °C for 3 days. The polar lipids of BXX^T were determined according to the methods of Chen *et al.* [17]. The major cellular fatty acids were $C_{16:0}$ (26.8 %), $C_{14:0}$ (22.8 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) (16.4 %) and $C_{16:1}\omega9c$ (10.7 %). Iso-$C_{15:0}$ (41.5 %), $C_{16:0}$ (23.2 %) and $C_{14:0}$ (11.9 %) were the major acids of *C. thermobutyricum* DSM 4928^T but the absence of $C_{15:0}$ in the isolate indicated that it was distinct from *C. thermobutyricum*. The main polar lipids of BXX^T were diphosphatidylglycerol, phosphatidylethanolamine, unidentified aminolipids, an unidentified phospholipid and unidentified lipids.

The G+C content of the genomic DNA was determined by thermal denaturation [18]. The DNA G+C content of BXX^T was 33.7 mol%, which was similar to that of *C. homopropionicum* DSM 5847^T (32.0±1.0 mol%), but lower than that of *C. thermobutyricum* DSM 4928^T (37.0 mol%) and *C. thermopalmarium* DSM 5974^T (35.7 mol%). It falls within the DNA G+C content range of the members of the genus *Clostridium* (23–37 mol%) [8].

The genome of BXX^T in its current assembly has a size of 6 959 731 bp. The DNA G+C content is 32.6 mol%. It contains 7138 predicted genes, and 158 tRNAs and 21 rRNA clusters are present. As expected for an endospore-forming bacterium, the gene for the master regulator of sporulation, Spo0A (scaffold2_gene836) was identified. As with all other clostridia, BXX^T does not carry genes encoding a phosphorelay (Spo0F and Spo0B), in contrast to the members of the genus *Bacillus*. The bacterium is motile by means of flagella. The relative gene *cheY* (scaffold11_gene3295, scaffold37_gene5570 and scaffold49_gene6016) is present. The BXX^T genome contains genes encoding enzymes of the methyl and carbonyl branches of the Wood–Ljungdahl pathway, including formate dehydrogenase (scaffold21_gene4409 and scaffold29_gene5113), formyl-THF synthetase *fhs* (scaffold35_gene5473, scaffold94_gene6472 and scaffold5_gene1921), formyl-THF cyclohydrolase *fchA* (scaffold10_gene3057 and scaffold22_gene4475), methylene-THF dehydrogenase *folD* (scaffold459_gene7119 and scaffold2_gene1165), methylene-THF reductase *met* (scaffold1_gene359), methyltransferase *acsE* (scaffold1_gene603), CO dehydrogenase *acsA* and *cooC* (scaffold21_gene4442 and scaffold1_gene379), acetyl-CoA synthase *acsB* (scaffold1_gene543), phosphotransacetylase (scaffold4_gene1758 and scaffold8_gene2758), and acetate kinase (scaffold26_gene48492 and scaffold135_gene6612).

Based on the results of this study, BXX^T represents a novel species of the genus *Clostridium*, with the proposed name *Clostridium bovifaecis* sp. nov.

DESCRIPTION OF *CLOSTRIDIUM BOVIFAECIS* SP. NOV.

Clostridium bovifaecis (bo.vi.fae′cis. L. fem. n. *faex*, *faecis* the dredge, faeces; L. masc. or fem. n. *bos*, *bovis*; N.L. gen. n. *bovifaecis* of cow manure).

Cells are Gram-staining-positive, motile, spore-forming, rod-shaped and strictly anaerobic. The isolate grows at 20–45 °C with optimum temperature of 30 °C. The pH range for growth at 30 °C is pH 5–10, with optimum pH at 7.0. Grows well in medium with 1 % NaCl and tolerates up to 2 % NaCl. Able to utilize cellobiose, D-glucose, D-mannose, maltose, sucrose, β-galactose, α-galactose, β-mannitol and maltotriose. No growth is observed on D-galactose, leucine, phenylalanine, L-proline, L-pyrrolydonyl, tyrosine, alanine–phenylalanine–proline, *N*-acetyl-D-glucosamine, β-glucose, urea, α-arabinose, arginine, β-D-fucose, phosphate, L-arabinose, D-ribose, phosphates, 5-bromo-4-chloro-3-hydroxyindoline-B-*N*-acetylglucosamine, α-L-fucose, α-L-arabinose, 5-bromo-4-chloro-3-indole-α-D-mannoside or D-xylose. The cells utilize H_2/CO_2, syngas ($H_2:CO_2:CO=4:3:3$), fructose, glycerol and other substances to generate acetate. The predominant cellular fatty acids are $C_{16:0}$, $C_{14:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:1}\omega9c$. The main polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, unidentified aminolipids, an unidentified phospholipid and unidentified lipids.

The type strain is BXX^T (=JCM 32382^T=CGMCC 1.5228^T), was isolated from cow manure. The DNA G+C content of the type strain is 33.7 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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