Note

Clostridium colicanis sp. nov., from canine faeces

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Morphological, biochemical and molecular genetic studies were performed on an unknown, anaerobic, rod-shaped organism isolated from faeces of a canine. The organism was tentatively identified as a member of the genus *Clostridium* based on its cellular morphology and ability to form endospores but, biochemically, it did not appear to correspond to any recognized species of this genus. Comparative 16S rRNA gene sequence analysis showed that the bacterium represents a previously unrecognized subline within *Clostridium* rRNA group I (*Clostridium sensu stricto*), which includes *Clostridium butyricum*, the type species of the genus. The nearest phylogenetic relatives of the unknown bacterium corresponded to *Clostridium absonum*, *Clostridium baratii*, *Eubacterium budayi*, *Eubacterium moniliforme*, *Eubacterium multiforme* and *Eubacterium nitritogenes*, but 16S rRNA sequence divergence values of > 3% demonstrated that it represents a novel species. Based on the findings presented, a novel species, *Clostridium colicanis* sp. nov., is described, with the type strain 3WC2^T (=CCUG 44556^T =DSM 13634^T).

Information on the intestinal microflora of canines, unlike that for humans, is currently extremely limited. However, from the limited investigations performed, it is evident that the canine colonic microbiota encompasses a diverse range of bacteria, consisting of many, predominantly anaerobic, genera and species (Balish *et al.*, 1977; Davis *et al.*, 1977). The major categories of organisms reported to be resident in the intestines and faeces of canines include bacteroides, bifidobacteria, clostridia, eubacteria, lactobacilli and anaerobic Gram-positive cocci (Balish *et al.*, 1977; Davis *et al.*, 1977; Benno & Mitsuoka, 1992; Benno *et al.*, 1992a), although, due to limitations of phenotypic methods of characterization, precise information on the nature of species present is often lacking. A large number of bacterial isolates recovered from canines have, in the past, been assigned to species on the basis of poor taxonomic evidence (Balish *et al.*, 1977; Davis *et al.*, 1977) whilst, in other cases, organisms remain unidentified and have simply been assigned to broad groups (e.g. clostridia, anaerobic Gram-positive cocci) (Mitsuoka, 1985; Benno & Mitsuoka, 1989, 1992; Benno *et al.*, 1992a, b). Consequently, it is highly likely that the canine gut harbours many organisms that do not correspond to currently defined species and represent hitherto unknown taxonomic diversity.

During the course of a molecular-based taxonomic study of the micro-organisms of canine faeces, we have isolated an unusual Gram-positive, spore-forming, rod-shaped organism that does not appear to correspond to any recognized *Clostridium* species based on phenotypic criteria. In this article, we report the phenotypic characteristics of this *Clostridium*-like organism and the results of a phylogenetic study. Based on these findings, we propose that the unknown, spore-forming, rod-shaped bacterium be designated as the type strain of a novel species of the genus *Clostridium*, *Clostridium colicanis* sp. nov.

A rod-shaped organism designated 3WC2^T was isolated from the faeces of a male Labrador dog. The faecal sample was collected immediately after defecation and was used to prepare a 10% (w/v) slurry using pre-reduced PBS (0.1 M phosphate, pH 7). The slurry was transferred into an anaerobic cabinet (H₂/CO₂/N₂, 10:10:80 by vol.) and homogenized for 10 min. Serial 10-fold dilutions were prepared using half-strength peptone water and cysteine hydrochloride (0.5 g l⁻¹). The strain was isolated from Wilkins–Chalgren agar for total anaerobes (Wilkins & Chalgren, 1976) incubated at 37 °C for 4 days, from a 10⁻⁴ dilution.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSM 13634^T is AJ420008.

Haemolysis was tested on Columbia sheep-blood agar (BBL). Motility was examined from peptone/yeast extract/glucose

(PYG) broth and agar, trypticase-nitrate broth (BBL) and Columbia sheep-blood agar (BBL). Temperature ranges for growth were determined in PYG medium (with 1 g Tween 80 l⁻¹ and 2 g sodium bicarbonate l⁻¹) (Holdeman *et al.*, 1977) using 100% N₂ as the atmosphere in Hungate tubes. Growth was recorded by measuring the OD₆₀₀ directly in Hungate tubes using an LKB Ultrospec II spectrophotometer. Traditional biochemical tests were performed as described by Holdeman *et al.* (1977). Hydrolysis of starch and gelatin was, in addition, tested using double-layer starch-agar plates and 0.4% (w/v) gelatin-agar plates. Biochemical tests were also performed using the commercially available API Rapid ID32AN system (bioMérieux) according to the manufacturer's instructions. Fermentation products formed after growth in peptone/yeast extract (PY) medium with 1% (w/v) glucose were determined as described by Holdeman *et al.* (1977). Alcohols and volatile acids were extracted with tert-butyl ethyl ether instead of diethyl ether. A Shimadzu GC-14A gas chromatograph, a Nukol capillary column (30 m) at 170 °C, a flame-ionization detector and hydrogen carrier gas were used for the analysis. For the determination of G + C content, DNA was isolated after disruption of cells using a French pressure cell and then purified in hydroxyapatite. The G + C content (mol%) was determined by HPLC after digestion of DNA with P1 nuclease and alkaline phosphatase as described by Mesbah *et al.* (1989). The 16S rRNA gene(s) of the isolate was amplified by PCR and sequenced directly using a dRhodamine terminator cycle sequencing kit (PE Applied Biosystems) and an automatic DNA sequencer (model 377; PE Applied Biosystems). The closest known relatives of the novel isolate were determined by performing database searches using the program FASTA. These sequences and those of other known related strains were retrieved from GenBank or the Ribosomal Database Project database and aligned with the newly determined sequence using the program DNATools (Rasmussen, 1995). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (Nicholas *et al.*, 1997). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR and the stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The faecal organism consisted of non-motile, spore-forming, rod-shaped cells. Typical cells were 0.9–1.0 × 3–10 µm. Spores were oval to oblong in PY-starch medium; the position of the spores varied from subterminal to almost terminal or occasionally central. The organism stained Gram-negative and was anaerobic, catalase-negative and produced acid from glucose as outlined in the species description. Results of classical biochemical tests and the API Rapid ID32AN test system are given in the species description below. Determination of the G + C content of DNA of strain 3WC2^T revealed a value of 31.7 mol%, indicating that it was a member of the low-G + C, Gram-positive *Clostridium* subphylum.

To ascertain the phylogenetic relationships of the unknown organism, comparative 16S rRNA gene sequencing was conducted. The partial sequence (>1400 nt) of the 16S rRNA gene of the isolate was determined. Sequence database searches revealed that the unknown bacterium from canine faeces was most closely related to clostridial species, in particular members of *Clostridium* rRNA group I (data not shown). Treeing analysis confirmed these findings, with the unidentified bacterium clustering with a small group of organisms within *Clostridium* rRNA group I that included *Clostridium absonum* (95.0% sequence similarity), *Clostridium baratii* (96.0%), *Eubacterium budayi* (95.5%), *Eubacterium moniliforme* (96.4%), *Eubacterium multiforme* (95.1%) and *Eubacterium nitritogenes* (95.1%). Fig. 1 depicts a neighbour-joining tree showing the phylogenetic position of the unidentified bacterium within *Clostridium* rRNA group I.

It is evident from the results of the taxonomic study that the spore-forming, rod-shaped isolate from the faeces of a canine represents a hitherto unknown species of the genus *Clostridium*. The genus *Clostridium*, as currently defined, harbours a phenotypically and phylogenetically diverse range of organisms. The taxonomy of the genus is further compounded by the fact that many non-spore-forming species (e.g. many *Eubacterium* species) are phylogenetically inter-dispersed with the clostridia. However, there is now good evidence that the genus *Clostridium* should be restricted to *Clostridium butyricum*, the type species of the genus, and its close phylogenetic relatives (Collins *et al.*,

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the nearest phylogenetic relatives of *C. colicanis* sp. nov. within *Clostridium* rRNA group I. The tree, constructed using the neighbour-joining method, was based on a comparison of 1353 nt. *Clostridium fervidus* (L09187) and *Clostridium pfennigii* (X77838) were used as an outgroup (not shown). Bootstrap values, expressed as percentages of 500 replications, are given at branching points. Database accession numbers are given in parentheses. Bar, 1% sequence divergence.

1994). *C. butyricum* and its close neighbours form a phylogenetically distinct group (designated *Clostridium* rRNA group I; Collins *et al.*, 1994), and the recovery, from a dog, of the novel bacterium within this rRNA cluster clearly demonstrates that it is, taxonomically, an authentic *Clostridium* species. Phylogenetically, the unidentified bacterium forms a distinct subline within rRNA group I, displaying a loose association with a small subcluster of species that includes *C. absonum*, *C. baratii*, *E. budayi*, *E. moniliforme*, *E. multiforme* and *E. nitritogenes*. Bootstrap resampling, however, showed that this association was not particularly significant. Sequence divergence values of approximately 3.5–5 % between the faecal isolate and the aforementioned species showed unequivocally that the canine bacterium merits classification as a distinct species. Furthermore, the canine bacterium is phenotypically different from other clostridial species. Based on both phenotypic and phylogenetic considerations, we therefore propose that the unknown, spore-forming, rod-shaped organism from dog faeces be classified in the genus *Clostridium*, as *Clostridium colicanis* sp. nov. Tests that are useful in distinguishing *C. colicanis* from its nearest relatives are shown in Table 1.

Description of *Clostridium colicanis* sp. nov.

Clostridium colicanis (co.li.can'is. L. n. *colum* colon, gut; L. gen. n. *canis* of the dog; N.L. gen. n. *colicanis* of the gut of a dog).

Cells are rod-shaped, approximately $0.9\text{--}1.0 \times 3\text{--}10\text{ }\mu\text{m}$ and stain Gram-negative. Spores are observed which are oval to oblong in PY-starch medium; position varies from subterminal to almost terminal or even central. Non-motile. Colonies are 3–5 mm in diameter, round, have an undulate

margin and are slightly convex, opaque, greyish-white and glossy. Non-haemolytic on Columbia sheep-blood agar. Growth occurs at 30 and 45 °C but not at 20 or 50 °C; optimum growth temperature approx. 37–40 °C. Anaerobic and catalase-negative. Produces acid from glucose (acidification of PY medium containing 1 % glucose, pH 6.8; after 6 days the pH was 4.8). Using traditional tests, acid is produced from cellobiose, aesculin (weak), fructose, galactose, glucose, lactose, maltose, mannose, ribose, salicin (weak), starch (weak) and sucrose. Acid is not produced from amygdalin, L-arabinose, glycogen, inositol, mannitol, melezitose, melibiose, raffinose, rhamnose, sorbitol, trehalose or xylose. Aesculin and urea, but not gelatin, are hydrolysed. Lecithinase- and lipase-negative. Indole is not produced. Nitrate is reduced to nitrite. Using the commercially available API Rapid 32AN test system, activity is detected for alkaline phosphatase, arginine arylamidase, arginine dihydrolase, β -galactosidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase and *N*-acetyl- β -glucosaminidase. No activity is detected for alanine arylamidase, α -arabinosidase, α -fucosidase, α -glucosidase, β -glucosidase, β -glucuronidase, α -galactosidase, β -galactosidase-6-phosphate, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, phenylalanine arylamidase, proline arylamidase, pyroglutamic acid arylamidase, serine arylamidase or tyrosine arylamidase. Nitrate reduction is not detected using the API Rapid ID32AN test system. The G + C content of the DNA is 31.7 mol%. The type strain is 3WC2^T (=DSM 13634^T =CCUG 44556^T). Isolated from faeces of a male Labrador dog.

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Table 1. Characteristics that are useful in distinguishing *C. colicanis* sp. nov. from its nearest phylogenetic relatives

Species: 1, *C. colicanis* sp. nov.; 2, *C. absonum*; 3, *C. baratii*; 4, *E. budayi*; 5, *E. moniliforme*; 6, *E. multiforme*; 7, *E. nitritogenes*. v, Variable.

Test	1	2	3	4	5	6	7
Spores	+	+	+	–	–	–	–
Motility	–	v	–	–	+	v	–
Lecithinase	–	+	+	+	v	–	v
Acid from:							
Cellobiose	+	+	+	+	–	v	+
Aesculin	+	+	+	–	–	–	–
Lactose	+	+	+	v	–	+	–
Maltose	+	+	+	+	v	–	+
Ribose	+	+	–	–	–	–	v
Salicin	+	+	+	v	–	–	v
Starch	+	–	v	v	v	–	v
Sucrose	+	+	+	–	–	–	v
Trehalose	–	+	–	–	–	–	v

and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.

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