Butyricicoccus pullicaecorum gen. nov., sp. nov., an anaerobic, butyrate-producing bacterium isolated from the caecal content of a broiler chicken

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Five isolates that produced large amounts of butyrate were obtained in the course of a study on the butyrate-producing microbiota from the caecal content of a 4-week-old broiler chicken. The five isolates were virtually indistinguishable in biochemical and genetic terms, suggesting that they were derived from a single bacterial clone colonizing this habitat. A phylogenetic analysis based on 16S rRNA gene sequences demonstrated that the five isolates represented a unique lineage within the *Clostridium leptum* subgroup of the clostridia, with *Eubacterium desmolans* as the closest phylogenetic neighbour (about 93 % similarity). These data indicate that the five novel isolates represent a single novel species within a novel genus, for which we propose the name *Butyricicoccus pullicaecorum* gen. nov., sp. nov. The type strain of *Butyricicoccus pullicaecorum* is 25-3^T (=LMG 24109^T =CCUG 55265^T). The DNA G+C content of strain 25-3^T was 54.5 mol%.

In broiler chickens, the gastrointestinal tract harbours numerous bacterial species, of which only 10 % have been characterized: 35 % represent previously unknown species within a known bacterial genus and the remaining 55 % represent bacteria for which even genus allocation is not possible (Apajalahti *et al.*, 2004). A study of the microbiota of chicken caeca conducted by Lan *et al.* (2002) showed the predominance (94 %) of sequences from within the phylum *Firmicutes*, the most abundant sequences belonging to the *Clostridium coccoides* subgroup and the *Clostridium leptum* subgroup (Collins *et al.*, 1994). Both subgroups contain numerous members that are known to produce butyrate as a product of fermentation (Pryde *et al.*, 2002). There is increased interest in butyrate-producing

strains in the context of veterinary medicine, because butyrate reduces invasion and colonization by *Salmonella* and stimulates intestinal epithelial cell growth (Van Immerseel *et al.*, 2005; Kien *et al.*, 2007). To our knowledge, butyrate-producing bacteria belonging to the phylum *Firmicutes* have not been isolated from the chicken gastrointestinal tract to date.

Bacteria were obtained from the caecal content of a 4-week-old broiler chicken that had been given a wheat-based, commercial, broiler feed supplemented with 2 % fructo-oligosaccharides (Orafti) from week 3. The chicken was euthanized and a sample of the caecal content was homogenized in anaerobic M2GSC medium (Miyazaki *et al.*, 1997) as described by Barcenilla *et al.* (2000). Tenfold serial dilutions of this suspension were made and, from each dilution, 0.3 ml was spread onto agar plates containing M2GSC medium with 1.5 % agar. The plates were incubated at 42 °C, which is similar to the body temperature of poultry (Richards, 1970), for 48 h and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 25-3^T is EU410376.

RAPD and REP-PCR genomic patterns and a scanning electron micrograph of cells of strain 25-3^T are available as supplementary figures with the online version of this paper.

then 58 single colonies were transferred to tubes containing M2GSC broth (10 ml). All manipulations were performed in an anaerobic workstation (Ruskinn Technology). Short-chain fatty acid concentrations in overnight cultures were analysed using GLC (GC14; Shimadzu) as described by Van Nevel & Demeyer (1977). Several butyrate-producing cultures were obtained. The supernatants of five of these bacterial cultures, i.e. ones obtained using isolates 11-3, 25-3^T, 44-3, 49-3 and 54-3, contained more than 13 mM butyric acid. These isolates were characterized in detail.

Repetitive-element-primed PCR with the (GTG)₅ primer and randomly amplified polymorphic DNA typing using two distinct primers (5'-TGCGCGCGGG-3' and 5'-AGCGGGCCAA-3') were performed as described previously (Versalovic *et al.*, 1994; Coenye *et al.*, 2002) after DNA extraction with an alkaline lysis procedure. The isolates yielded identical, or virtually identical, DNA fingerprints in all three assays (see Supplementary Figs S1 and S2, available in IJSEM Online), suggesting that all five originate from a single strain that colonized the intestinal tract of the sampled chicken.

To elucidate the phylogenetic position of the five isolates, almost-complete 16S rRNA gene sequences (corresponding to positions 8–1541 in the *Escherichia coli* numbering system) were determined for each isolate by using the 'universal' eubacterial primers fD1 and rD1 (Weisburg *et al.*, 1991). The purified amplicons were sequenced using the BigDye Terminator sequencing kit with primers pD, Gamma*, 3 and O* on an ABI PRISM 310 Genetic Analyzer (Coenye *et al.*, 1999). The closest match to the deduced sequences was found using the FASTA program (Pearson & Lipman, 1988). The nucleotide sequences were aligned with reference 16S rRNA gene sequences by using the CLUSTAL W program (Thompson *et al.*, 1994) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) via the PHYLIP package (Felsenstein, 1989), using DNADIST for distance analysis (Kimura, 1980) (Fig. 1).

Fig. 1 shows the phylogenetic position of the five isolates among their closest relatives, i.e. members of the *C. leptum* subgroup. The isolates shared virtually identical (98–99%) 16S rRNA gene sequences and were most closely related to a butyrate-producing bacterium from the human gut (Barcenilla *et al.*, 2000) (94% sequence similarity) and to *Eubacterium desmolans* ATCC 43058^T (Morris *et al.*, 1986) (93%).

Determination of the genomic DNA G+C content of isolate 25-3^T was performed by using a Waters Breeze HPLC system and an XBridge Shield RP18 column maintained at 37 °C. This gave a value of 54.5 mol%.

All five isolates comprised Gram-positive coccoid cells that were 1.2–1.5 µm long. A scanning electron micrograph of isolate 25-3^T is shown in Supplementary Fig. S3. Following overnight incubation on M2GSC medium at 42 °C, colonies were white in colour and 1–2 mm in diameter.

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the five butyrate-producing isolates and members of the *C. leptum* subgroup. The tree was constructed using the neighbour-joining method and was based on a comparison of approximately 1360 nt. Numbers at nodes are bootstrap percentages (based on 100 resamplings); values <80% are not shown. The 16S rRNA gene sequence of *Clostridium perfringens* ATCC 13124^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

Characterization of the biochemical properties of the strains was done using the commercially available API 20A microtest system (bioMérieux) according to the manufacturer's instructions, except that incubation was performed at 42 °C instead of 36 ± 2 °C. All five isolates showed hydrolysis of aesculin and fermentation of glucose, salicin, sucrose, maltose, xylose, cellobiose, mannose and trehalose. No urease activity was detected and hydrolysis of gelatin and fermentation of mannitol, lactose, arabinose, glycerol, melezitose, raffinose, sorbitol and rhamnose were absent. The ability of the isolates to degrade starch was determined by culturing them on M2GSC agar, which contains 0.2% starch. After overnight anaerobic incubation at 42 °C, the plates were flooded with a commercial Lugol solution (Sigma). Clear zones around areas of cell growth indicated hydrolysis of starch.

When plates inoculated with isolate 25-3^T were exposed to air for more than 3.5 h, subsequent anaerobic growth seemed to be prevented. The isolates produced more than 13 mM butyrate and utilized acetate; three out of the five isolates consumed small amounts of propionate. Four strains were also tested for H₂ and CO₂ production. For this purpose, 1 ml gas phase was sampled with a gas-tight syringe and analysed for H₂ and CO₂ (FM Dual Column GC 700; Avondale) (Van Nevel *et al.*, 1970) (Table 1).

In conclusion, in the course of a study on the butyrate-producing microbiota from the caecal content of a 4-week-old broiler chicken, five isolates were obtained that produced large amounts of butyrate in overnight cultures of M2GSC broth. The five isolates were virtually indistinguishable in biochemical and genetic terms, suggesting that they were derived from a single bacterial clone colonizing

Table 1. Fermentation products of the five caecal isolates

The isolates were grown overnight in M2GSC broth at 42 °C. Concentrations (mM) are means of three replications ($\pm$ SD). ND, Not done.

Isolate	Acetate	Propionate	Butyrate	H ₂	CO ₂
11-3	-10.6 $\pm$ 4.3	-0.6 $\pm$ 1.1	13.2 $\pm$ 1.0	ND	ND
25-3 ^T	-14.3 $\pm$ 1.7	-1.4 $\pm$ 1.2	18.6 $\pm$ 1.2	6.6 $\pm$ 0.3	16.5 $\pm$ 4.7
44-3	-14.6 $\pm$ 3.5	-1.1 $\pm$ 0.3	15.5 $\pm$ 4.3	7.9 $\pm$ 0.9	17.7 $\pm$ 1.2
49-3	-12.4 $\pm$ 0.7	-0.7 $\pm$ 0.4	18.6 $\pm$ 5.1	7.1 $\pm$ 0.7	13.6 $\pm$ 2.8
54-3	-11.8 $\pm$ 5.9	-0.2 $\pm$ 0.9	13.6 $\pm$ 0.2	5.8 $\pm$ 1.8	15.6 $\pm$ 1.0

this habitat. A phylogenetic analysis demonstrated that the five isolates represented a unique lineage within the phylum *Firmicutes*, with *E. desmolans* ATCC 43058^T as the closest phylogenetic neighbour. These data indicate that the five novel isolates represent a single novel species within a novel genus, for which the name *Butyricoccus pullicaecorum* gen. nov., sp. nov. is proposed.

The closest phylogenetic neighbour, *E. desmolans*, can be readily distinguished from *B. pullicaecorum* sp. nov. on the basis of biochemical criteria. In contrast to *B. pullicaecorum*, *E. desmolans* does not ferment cellobiose, aesculin, glucose, maltose, mannose or trehalose. Reclassification of *E. desmolans* as a member of the genus *Butyricoccus* cannot be considered, as there is only 93 % 16S rRNA gene sequence similarity between the two type strains and their G+C contents are very different (35 mol% for *E. desmolans* ATCC 43058^T versus 54.5 mol% for *B. pullicaecorum* 23-5^T).

Description of *Butyricoccus* gen. nov.

Butyricoccus (Bu.ty.ri'ci.coc'cus. N.L. n. *acidum* butyricum butyric acid; N.L. masc. n. *coccus* a coccus; N.L. masc. n. *Butyricoccus* coccoid-shaped bacterium that produces butyrate).

Cells are Gram-positive, anaerobic, non-motile, coccoid and usually arranged in pairs (occasionally in short chains). Produce butyrate, H₂ and CO₂ and utilize acetate in M2GSC broth. The phylogenetic position is in a novel lineage in the class *Clostridia* of the phylum *Firmicutes*. The type species is *Butyricoccus pullicaecorum*.

Description of *Butyricoccus pullicaecorum* sp. nov.

Butyricoccus pullicaecorum (pul.li.cae.co'rum. L. n. *pullus* a chicken; L. n. *caecum* caecum; L. gen. pl. n. *caecorum* of caeca; N.L. gen. pl. n. *pullicaecorum* of the caeca of chickens).

Displays the following properties in addition to those described for the genus. Cells are approximately 1.2–1.5 µm long. On M2GSC agar after 24 h at 42 °C, colonies are white, smooth, circular and 1–2 mm in diameter. All five known isolates hydrolyse aesculin and ferment glucose,

salicin, sucrose, maltose, xylose, cellobiose, mannose and trehalose. Urease activity, gelatin hydrolysis and fermentation of mannitol, lactose, arabinose, glycerol, melezitose, raffinose, sorbitol and rhamnose are absent. The G+C content of genomic DNA of the type strain is 54.5 mol%.

The type strain, 25-3^T (=LMG 24109^T =CCUG 55265^T), was isolated in Ghent (Belgium) in 2007 from the caecal content of a 4-week-old broiler chicken.

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