

NOTE

***Clostridium lactatifermentans* sp. nov., a lactate-fermenting anaerobe isolated from the caeca of a chicken**

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An obligately anaerobic, lactate-fermenting bacterium (strain G17^T) was isolated from the caeca of a 31-day-old chicken. Grown at neutral pH, cells were rod-shaped with tapered ends and showed no motility and no spore formation. Electron microscopy showed that the cell walls had a Gram-positive structure. The DNA G+C content was 44.6 mol%. Based on 16S rDNA sequence analysis, strain G17^T was considered to belong to the low-G+C-content Gram-positive bacteria of cluster XIV subgroup b and most closely related to *Clostridium propionicum* (93.5%) and *Clostridium neopropionicum* (93.5%). The optimum temperature for growth was 41 °C and the optimum pH was pH 6.4–7.3. The optimum temperature of 41 °C suggests that strain G17^T might have become adapted to the body temperature of chickens. Strain G17^T was able to grow on a variety of organic compounds. Most of these compounds were converted to acetate, propionate and traces of butyrate and isovalerate. In media with mixtures of substrates, lactate was degraded by strain G17^T before the other substrates. This indicates that strain G17^T might be important in the fermentation of lactate in the caeca of chickens. Based on its physiological and phylogenetic characteristics, it is proposed that strain G17^T should be assigned to the genus *Clostridium* as a novel species, *Clostridium lactatifermentans* sp. nov.

Keywords: *Clostridium lactatifermentans* sp. nov., lactate fermentation, caeca of chickens

Chickens are animals with hindgut fermentation and lactate may therefore be an important substrate for bacteria in the caeca of chickens. This idea is supported by the fact that lactate concentrations are low in the caeca of chickens, while lactate-producing bacteria such as lactobacilli and bifidobacteria are present in large numbers (van der Wielen *et al.*, 2000). In other animals with hindgut fermentation, it has been shown that lactate is an important substrate for fermentation (Stevani *et al.*, 1991). Bacteria mostly associated with

intestinal lactate fermentation are propionibacteria, *Veillonella* species and *Megasphaera elsdenii* (Hinton & Hume, 1995; Stevani *et al.*, 1991). During the investigation of chicken caecal bacteria capable of fermenting lactate, we isolated an unknown bacterium that was designated strain G17^T. This strain was phylogenetically and phenotypically related to *Clostridium propionicum* and *Clostridium neopropionicum*. Based on phylogenetic and phenotypic evidence, we propose the classification of the bacterium as *Clostridium lactatifermentans* sp. nov.

Strain G17^T was isolated from the caeca of a broiler chicken (31 days old) raised in a commercial farmhouse in Leusden, The Netherlands. Caecal material from this broiler chicken was diluted in physiological reduced salt solution (8 g NaCl l⁻¹; 0.5 g cysteine hydrochloride l⁻¹) under anaerobic conditions. Dilu-

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The GenBank accession number for the 16S rDNA sequence of strain G17^T is AY033434.

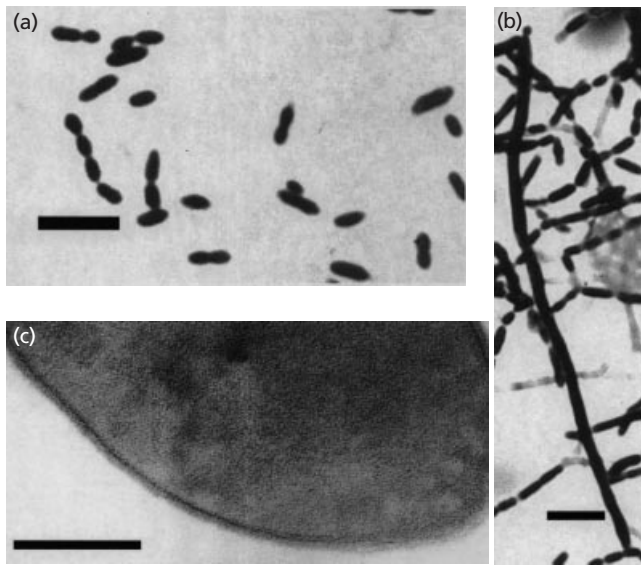


Fig. 1. (a)–(b) Photomicrographs of Gram-stained cells of strain G17^T grown in BHI broth at pH 6.8 (a) and pH 5.6 (b); bars, 10 μ m. (c) Electron micrograph of thin-sections of strain G17^T showing the single-layered structure of the cell wall; bar, 200 nm.

tions were spread-plated on a mineral carbonate-buffered medium (van der Wielen *et al.*, 2001) supplemented with 1.5% agar and DL-lactate (concentration 40 mM). Plates were incubated at 37 °C in an anaerobic chamber (80% N₂, 15% CO₂, 5% H₂) for 4 days. Several colonies were obtained as pure cultures by repeated streaking on the above-mentioned agar medium. One strain (G17^T) was studied in more detail. Reference strains of *C. propionicum* (DSM 1682^T) and *C. neopropionicum* (DSM 3847^T) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were cultivated according to protocols from the supplier. For the description of cell morphology, strain G17^T was grown under anaerobic conditions in brain/heart infusion (BHI) broth containing 0.5 g cysteine hydrochloride l⁻¹ at pH 5.6 and 6.8. Examination was done using a light microscope (Zeiss) in phase-contrast mode and with a light microscope (Zeiss) after Gram-staining. Exponentially growing cells (at 37 °C for 18 h) were Gram-stained (Mossel & Jacobs-Reitsma, 1990). Gram-type was also determined by the KOH test (Powers, 1995). Transmission electron microscope examination was done as described by Plugge *et al.* (2000). To test for sporulation, a 48-h-old culture of strain G17^T (BHI broth with 0.5 g cysteine hydrochloride l⁻¹) was placed at 70 or 80 °C for 10 min. Subsequently, the heat-treated cultures were streaked on BHI agar and incubated at 37 °C in an anaerobic chamber for 48 h. Growth rates were determined by measuring the increase in OD₆₀₀ over time with a spectrophotometer (Pharmacia). BHI broth containing 0.5 g cysteine hydrochloride l⁻¹ was adjusted with

1 M HCl or 1 M NaOH to 14 different pH values ranging from 5.2 to 8.6 in duplicate. Growth rates were determined for nine different temperatures ranging from 25 to 49 °C in duplicate. The temperature range for growth was determined at pH 7.0 in the same medium as described for the pH. Broths were inoculated with an overnight-grown culture of strain G17^T (BHI broth, pH 7.0 at 37 °C) and incubated anaerobically for up to 4 weeks.

All biochemical tests were performed in duplicate with strain G17^T, *C. propionicum* and *C. neopropionicum*. Substrate utilization was determined in mineral carbonate-buffered medium at 37 °C and an initial pH of 7.0 (van der Wielen *et al.*, 2001). Substrates tested were glucose, xylose, pyruvate, DL-lactate, succinate, acrylate, L-alanine, L-cysteine, L-serine, L-threonine and ethanol for the two clostridial species and strain G17^T. Additionally, growth of strain G17^T was tested with cellobiose, melibiose, raffinose, arabinose, lactose, starch, sorbitol, L-valine, L-leucine, L-isoleucine, L-proline, L-lysine, L-aspartate, L-arginine and glycine. All substrates were tested at a concentration of 30 mM except for acrylate and ethanol, which were tested at both 30 and 15 mM. Fermentation products were analysed by HPLC (van der Wielen *et al.*, 2000). Furthermore, API 20A tests (bioMérieux) were used for substrate utilization of 16 sugars, gelatin hydrolysis, indole production, aesculin hydrolysis and urea hydrolysis. Catalase was tested on cells grown on BHI agar for 24 and 48 h.

For SDS-PAGE analysis of whole-cell proteins, strain G17^T and both reference strains were grown in BHI with 0.5 g cysteine hydrochloride l⁻¹ for 24 h. Cells were harvested by centrifugation (6000 g, 15 min) and then washed and pellets were resuspended in 1 ml physiological salt solution. Samples were diluted in denaturing loading buffer and boiled for 10 min. SDS-polyacrylamide running gels with 12% acrylamide and 4.5% acrylamide stacking gels were run in a Bio-Rad Mini Protean II system at 20 mA for 2 h. Aliquots of 20 μ l of each protein sample were loaded into each lane. Low-range molecular-mass markers for SDS-PAGE were obtained from Bio-Rad. The gels were stained overnight with 0.5% Coomassie brilliant blue R250 in ethanol/deionized water/acetic acid (4.5:4.5:1, by vol.) and destained for 3 h in ethanol/deionized water/acetic acid (4.5:4.5:1). The G + C content of the DNA was determined by HPLC (Logan *et al.*, 2000; Mesbah *et al.*, 1989). The 16S rRNA gene of strain G17^T was amplified by PCR (corresponding to positions 8–1510 of the *Escherichia coli* 16S rRNA gene). Both strands were sequenced directly using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (Applied Biosystems; model ABI prism 310). The sequence obtained (1497 bp) was aligned using the ARB software package (Strunk & Ludwig, 1991) and checked manually using the secondary structure. Obscurities in the alignment were checked for reading errors in the original sequence. BLAST homology searches were

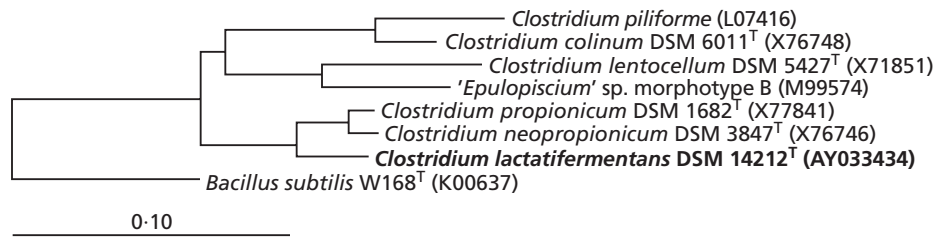


Fig. 2. Dendrogram based on 16S rDNA sequences showing the phylogenetic position of strain G17^T among representatives of the genus *Clostridium*, using the neighbour-joining method for calculation. *Bacillus subtilis* was used as the outgroup for the phylogenetic tree. Bar, 0.1 (evolutionary distance). All strains of *Clostridium* belonging to the low-G+C-content Gram-positive bacteria of cluster XIV subgroup b are shown, with GenBank accession numbers in parentheses.

performed using the GenBank and EMBL databases. Results of these searches were compared with the closest relatives found using the ARB software package. The ARB software package was also used for phylogenetic analysis. Least-squares distance-matrix analysis based on evolutionary distances was performed using the correction of Felsenstein. A neighbour-joining tree was constructed with the closest relatives.

Agar plates, with lactate as the sole carbon source, inoculated with a 10⁶ dilution of caecal material showed separate colonies, of which several were obtained as pure cultures. One of these cultures was designated strain G17^T and studied further. Cells of strain G17^T growing at pH 6.8 and 37 °C were rod-shaped with tapered ends. The cell size was 2.8–10 × 1.1–1.3 µm and cells occurred singly, in pairs and in chains (Fig. 1a). Cells elongated when growing at low pH (5.8) (Fig. 1b). Spores were never observed by phase-contrast microscopy and no growth occurred after cultures were heat-treated for 10 min at 70 or 80 °C. Cells stained Gram-negative but the KOH reaction was always negative (i.e. characteristic of Gram-positive cells). The cell-wall ultrastructure resembled that of Gram-positive bacteria (Fig. 1c). Growth of strain G17^T occurred under anaerobic conditions. No growth was observed under aerobic or microaerophilic (3–5% O₂) conditions.

The G+C content of strain G17^T was 44.6%. The nucleotide sequence (1497 bp) of the 16S rRNA gene was sequenced and revealed that strain G17^T was phylogenetically most closely related to the low-G+C-content Gram-positive bacteria of cluster XIV subgroup b (Collins *et al.*, 1994). This subgroup contains six different species with three different sublines, which probably represent distinct genera (Collins *et al.*, 1994). Sequence analysis showed that the most closely related bacterial species to strain G17^T are *C. propionicum* (93.5% sequence similarity) and *C. neopropionicum* (93.5%). A phylogenetic tree showing the relationship of strain G17^T to other species in cluster XIV of the clostridia is shown in Fig. 2. Total soluble cell proteins were separated by SDS-PAGE; the protein profiles of strain G17^T, *C. propionicum* and *C.*

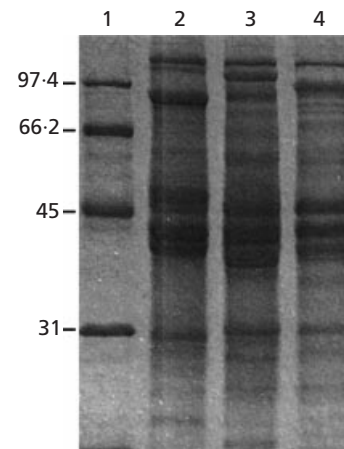


Fig. 3. SDS-PAGE of whole-cell proteins of strain G17^T and the closely related *C. propionicum* and *C. neopropionicum*. Lanes: 1, low-range protein standard; 2, strain G17^T; 3, *C. propionicum*; 4, *C. neopropionicum*.

neopropionicum showed distinguishable differences (Fig. 3).

Strain G17^T is a strictly anaerobic and chemo-organotrophic bacterium. Growth occurred between pH 5.6 and 8.3 with optimum growth between pH 6.4 and 7.3. The optimal temperature for growth was 41 °C and growth occurred between 30 and 47 °C. This differs from the phylogenetically and phenotypically related *C. propionicum* and *C. neopropionicum*, which have optimum temperatures of 30 °C and can grow at up to 40 °C (Cardon & Barker, 1946; Tholozan *et al.*, 1992). The pH optimum for strain G17^T was comparable to those of the related clostridia, but strain G17^T was also able to grow at pH 5.6, whereas *C. propionicum* did not grow below a pH value of 5.8 and *C. neopropionicum* below pH 6.1 (Cardon & Barker, 1946; Tholozan *et al.*, 1992). This indicates that strain G17^T might have become adapted to the caeca of chickens, where the temperature is 41 °C and the pH ranges from 5.6 to 6.5 (van der Wielen *et al.*, 2000). In contrast, the two related clostridia are not associated with the intestines

Table 1. Characteristic differences between strain G17^T, *C. propionicum* and *C. neopropionicum*

Data were taken from Cardon & Barker (1946), Tholozan *et al.* (1992) and this study. +, Positive; -, negative; ±, variable. All three taxa were Gram-positive by the KOH method.

Characteristic	Strain G17 ^T	<i>C. propionicum</i>	<i>C. neopropionicum</i>
Utilization of:			
Glucose	+	-	-
Xylose	+	-	-
Ethanol (30 mM)	-	+	+
Ethanol (15 mM)	-	+	+
Acrylate (30 mM)	-	+	-
Acrylate (15 mM)	-	+	+
API 20A test results:			
Glucose	+	-	+
Xylose	+	-	-
Motility	-	+	Slight
Shape	Rods, single or chains	Rods, single or chains	Rods, in pairs
Gram stain	-	±	-
Spore formation	-	+	+
Temperature for growth (°C):			
Optimum	41	30	30
Range	30-47	28-37	15-40
pH range for growth	5.6-8.5	5.8-8.6	6.1-8.2
Habitat	Caeca of chicken	Black mud	Industrial anaerobic digester
DNA G+C content (mol %)	44.6	36.0	34.5

of animals but were respectively isolated from marine sediments and from a mesophilic industrial anaerobic digester treating vegetable cannery wastewater (Cardon & Barker, 1946; Janssen, 1991; Tholozan *et al.*, 1992).

Glucose, xylose, DL-lactate, pyruvate, L-alanine, L-cysteine, L-serine and L-threonine were fermented by strain G17^T. Slow and moderate growth was observed with L-valine, L-leucine, L-isoleucine and L-aspartate. Cellobiose, melibiose, raffinose, lactose, arabinose, starch, sorbitol, succinate, ethanol (15 and 30 mM), acrylate (15 and 30 mM), L-proline, L-lysine, L-arginine and glycine were not utilized. Strain G17^T converted the utilized substrates, with the exception of L-threonine, mainly into high concentrations of acetate and propionate and trace amounts of butyrate and isovalerate. L-Threonine was fermented to high concentrations of propionate and butyrate with trace amounts of acetate and isovalerate. The capacity to ferment lactate may be an important characteristic of strain G17^T. Lactate is probably produced in high concentrations in the caeca of chickens, since lactate-producing bacteria are present in large numbers in the caeca (van der Wielen *et al.*, 2000). Since strain G17^T was among the dominant bacteria isolated on lactate as the sole carbon and energy source, it can be assumed that this strain is important for the fermentation of lactate in the caeca of broiler chickens. This is con-

firmed by the observation that, if strain G17^T was grown in a medium with mixed substrates of lactate and glucose or lactate and xylose, lactate was degraded preferably above the other component (data not shown). Strain G17^T differed from the two related clostridia in its inability to ferment ethanol or acrylate (Table 1). In previous reports, it was shown that *C. neopropionicum* was unable to use acrylate and only high cell densities dismutated acrylate to propionate and acetate (Tholozan *et al.*, 1992). This is in accordance with our finding that, at acrylate concentrations above 15 mM, *C. neopropionicum* was unable to grow. However, at concentrations of 15 mM or lower, *C. neopropionicum* can grow on acrylate, in contrast to strain G17^T. Strain G17^T was capable of using glucose or xylose as the sole substrate, whereas *C. neopropionicum* and *C. propionicum* could not (Table 1). According to the literature, *C. neopropionicum* should be capable of using glucose and xylose as a substrate (Tholozan *et al.*, 1992). This difference can be related to the use of different media for growth incubations. Under the growth conditions used in this study, the inability of *C. neopropionicum* to use glucose differed from strain G17^T.

Using the API 20A test, only the sugars glucose and xylose gave positive reactions for strain G17^T. Furthermore, gelatin was hydrolysed by strain G17^T but indole, urease, aesculin hydrolysis and catalase reac-

tions were negative. In the API 20A test, strain G17^T differed from *C. propionicum* in reducing the pH with glucose and xylose as substrate and differed from *C. neopropionicum* in reducing the pH with xylose as substrate. Other differences observed were the absence of endospores and the non-motility of strain G17^T compared with *C. propionicum* and *C. neopropionicum* (Table 1).

On basis of these phenotypic, genotypic and phylogenetic differences, we propose that strain G17^T should be assigned as a novel member of cluster XIV subgroup b of the low-G+C-content Gram-positive bacteria (Collins *et al.*, 1994).

Description of *Clostridium lactatifermentans* sp. nov.

Clostridium lactatifermentans (lac.ta'ti.fer.men.tans. N.L. n. *lactatum* lactate; L. part. adj. *fermentans* fermenting; N.L. adj. *lactatifermentans* fermenting lactate).

Rods with tapered ends that are 2.8–10 µm long and 1.1–1.3 µm wide. Stains Gram-negative but has a Gram-positive-type cell wall. Non-motile, non-spore-forming and strictly anaerobic chemo-organoheterotroph. Utilizes glucose, xylose, pyruvate, DL-lactate, L-alanine, L-serine, L-cysteine and L-threonine. Produces acetate, propionate, butyrate and isovalerate from lactate and glucose. Slow and moderate growth observed with L-valine, L-leucine, L-isoleucine and L-aspartate as substrate. No growth occurs on cellobiose, melibiose, raffinose, sorbitol, lactose, arabinose, starch, succinate, ethanol (15 or 30 mM), acrylate (15 or 30 mM), L-proline, L-lysine, L-arginine or glycine. Gelatin is hydrolysed whereas urease and aesculin are not. Cells are catalase-negative and indole is not produced. Grows optimally at 41 °C with a temperature range for growth of 30–47 °C. The pH range for growth is 5.6–8.3 with optimum growth at pH 6.4–7.3. The DNA G+C content is 44.6%. The type strain, G17^T (= DSM 14214^T = LMG 20954^T), was isolated from the caeca of a 31-day-old broiler chicken.

Acknowledgements

We want to thank Eric Spek for performing the electron microscopy, Bart Jordi, Corrie Zwaagstra and Erwin Zoetendal for help with the 16S rDNA sequencing and David Keukenkamp for performing the SDS-PAGE on soluble

proteins. Furthermore, we thank Bruno Pot for critical discussions and Caroline Plugge and Erwin Zoetendal for their advice and help with the manuscript.

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