

Campylobacter cuniculorum sp. nov., from rabbits

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Eight strains of an unknown thermotolerant *Campylobacter* species were isolated from the caecal contents of rabbits (*Oryctolagus cuniculus*). All strains were initially identified as belonging to the genus *Campylobacter* by means of genus-specific PCR, but none were identified using species-specific PCR for known thermophilic species. Cells were spiral shaped with bipolar unsheathed flagella, with no periplasmic fibres, and appeared coccoid after 10–12 days of incubation. Phylogenetic analyses based on 16S rRNA gene, *rpoB* and *groEL* sequences revealed that all strains formed a robust clade that was very distinct from recognized *Campylobacter* species. 16S rRNA gene sequence pairwise comparisons of strain 150B^T with the type strains of other *Campylobacter* species revealed that the nearest phylogenetic neighbour was *Campylobacter helveticus* NCTC 12470^T, with 96.6 % similarity. The uniqueness of these rabbit isolates was confirmed by whole-cell protein electrophoresis. Taken together, these data indicate that the strains belong to a novel *Campylobacter* species for which the name *Campylobacter cuniculorum* sp. nov. is proposed, with 150B^T (=LMG 24588^T =CCUG 56289^T) as the type strain.

The genus *Campylobacter* was proposed by Sebald & Véron (1963). In the following decades, this genus has expanded with the description of species originating from mammals and birds and now, after various reclassifications, includes 18 established species and six subspecies (Foster *et al.*, 2004; Vandamme *et al.*, 2005; Inglis *et al.*, 2007). So far, the few reports on the isolation of campylobacters from rabbits include strains of *Campylobacter jejuni* (Prescott & Bruin-Mosch, 1981; Weber *et al.*, 1982) and a *Campylobacter*-like organism (Reynaud *et al.*, 1993) from healthy and diarrhoeic animals. In the present paper, we describe the results of a polyphasic taxonomic investigation of eight strains of a *Campylobacter*-like organism recovered from rabbits (*Oryctolagus cuniculus*) in Italy.

Eight *Campylobacter*-like unidentified isolates were recovered from the caecal contents of eight rabbits during routine

bacteriological analysis. The isolates were obtained between 2005 and 2007 from animals reared in intensive and extensive farms in different regions, thereby representing a temporally, geographically and epidemiologically independent set of isolates. Isolations were made after 6–8 days of incubation at 37 °C in a microaerobic atmosphere with hydrogen, on nutrient sheep-blood agar [nutrient broth No. 2 (Oxoid) with 1.5 % Bacto agar (Difco) and 5 % sheep blood] plus cefoperazone, amphotericin B, teicoplanin selective supplement (CAT; Oxoid), on modified-charcoal cefoperazone deoxycholate agar (CM0739; Oxoid) and on nutrient sheep-blood agar using a filter method (Zandoni *et al.*, 2007). The microaerobic atmosphere with hydrogen was obtained by the gas replacement method using an anaerobic gas mixture (10 % H₂, 10 % CO₂, 80 % N₂) as described by Bolton *et al.* (1992).

After 6 days of incubation on nutrient sheep-blood agar, colonies were 1–2 mm in diameter, grey-green, flat with rough margins and slightly mucoid-looking; sometimes the colonies were α -haemolytic and exhibited a tailing effect along the streak line. Cells were Gram-negative, pleomorphic, typically sigmoid to allantoid in shape, 2.6 ± 0.7 μ m (mean \pm SD) in length and 0.3 ± 0.1 μ m in width when observed after Gram-staining. Cells appeared coccoid after 10–12 days of incubation.

Bacterial DNA was extracted by using a ChargeSwitch gDNA Mini bacteria kit (Invitrogen). The strains were

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *groEL* gene sequences of strains 150B^T, 117/07 and 120/07 are DQ400345, EU636818 and EU636820 (16S rRNA gene), EU636830, EU636833 and EU636836 (*rpoB*) and EU636828, EU636824 and EU636827 (*groEL*), respectively.

Neighbour-joining dendrograms based on *rpoB* and *groEL* partial gene sequences are available as supplementary material with the online version of this paper.

identified as *Campylobacter* using the genus-specific PCR described by Linton *et al.* (1996), but were not identified at the species level using species-specific PCR tests for *Campylobacter coli* and *C. jejuni* (Denis *et al.*, 1999), for *C. upsaliensis* and *C. helveticus* (Lawson *et al.*, 1997) or for *C. lari* (Linton *et al.*, 1996).

In order to establish the taxonomic position of the rabbit isolates, a phylogenetic analysis based on the sequences of the 16S rRNA gene was carried out. The nearly complete 16S rRNA gene was amplified using universal primers p27f (5'-AGAGTTTGATCCTGGCTCAG-3') and p1492r (5'-TACGGCTACCTTGTTACGACT-3') and the PCR-amplified template was sequenced by primer walking (Primm SRL). Sequences were assembled with VECTOR NTI software (Invitrogen) and then aligned in BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) with CLUSTAL W using publicly available *Campylobacter* reference sequences. The alignment was adjusted visually, removing intervening sequence regions and unknown bases, and data were corrected for multiple base changes by the method of Jukes & Cantor (1969). A phylogenetic tree was constructed in MEGA3 (<http://www.megasoftware.net/>) using the neighbour-joining method. Bootstrap analysis was performed with 1000 reassembled datasets.

A fragment of 1283 bp of the 16S rRNA gene was sequenced from each strain and a search of the NCBI database using MEGABLAST (<http://www.ncbi.nlm.nih.gov/blast/>) determined that the strains were most closely related to taxa within the genus *Campylobacter*, confirming the results from the genus-specific PCR. Pairwise comparisons of 16S rRNA gene sequences showed that the rabbit isolates were genetically highly related to each other, exhibiting

99.1–100 % sequence similarity. Furthermore, the neighbour-joining dendrogram (Fig. 1) indicated that all eight strains formed a robust clade (100 % bootstrap support) that was clearly distinct from all other *Campylobacter* species. Pairwise sequence comparisons of strain 150B^T with the type strains of the most closely related species revealed similarities of 96.6, 96.5 and 96.1 % with *C. helveticus* NCTC 12470^T, *C. jejuni* NCTC 11351^T and *C. upsaliensis* CCUG 14913^T, respectively.

In view of the low 16S rRNA gene sequence divergence between the unidentified strains and other *Campylobacter* species, the phylogenetic relationships were further examined by *rpoB* (Korczak *et al.* 2006) and *groEL* (Kärenlampi *et al.*, 2004) sequence analysis. Sequences were processed as described above. Phylogenetic trees based on partial nucleotide sequences of *rpoB* and *groEL* from eight strains and reference *Campylobacter* strains are shown in Supplementary Fig. S1 (available in IJSEM Online). In both trees, all of the unidentified strains clustered together in a tight clade clearly separated from all other *Campylobacter* species (100 % bootstrap support). The *rpoB* sequence similarity values within the clade of the rabbit strains were 97.9–100 %, while the similarity values towards the other *Campylobacter* species were 60.5–80.5 %. Likewise, *groEL* sequence similarity values among the rabbit strains were 97.7–100 % and values between strain 150B^T and other *Campylobacter* species were below 86 %. Similarly to Korczak *et al.* (2006) and Kärenlampi *et al.* (2004), we observed good congruence between *rpoB*, *groEL* and 16S rRNA gene sequence results, since each of the phylogenetic trees showed a similar topology. However, compared to the 16S rRNA gene sequence analysis, the *rpoB* and *groEL* sequence analysis showed lower interspecies similarity.

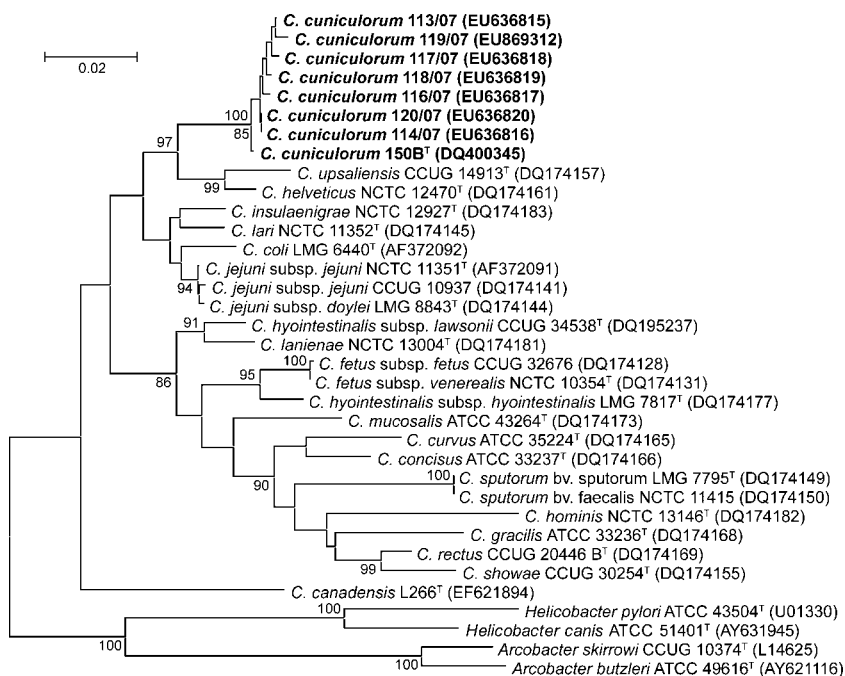


Fig. 1. Unrooted tree based on 16S rRNA gene sequences showing the relationships of the eight strains of *Campylobacter cuniculorum* sp. nov. with related species. Numbers at nodes (≥ 85 %) indicate support for internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps). Bar, 0.02 nucleotide substitutions per base.

Although all sequence data demonstrated that the eight isolates represent a coherent taxon, whole-cell protein electrophoresis was used to examine further the relationships between the isolates. Whole-cell protein electrophoresis was performed after culturing strains on Mueller–Hinton agar supplemented with 5 % horse blood at 37 °C for 48 h under microaerobic conditions with hydrogen. Protein extraction and SDS-PAGE were performed as described by Pot *et al.* (1994). Similarity between the normalized whole-cell protein patterns was determined by the Pearson product–moment correlation coefficient, after which clustering was performed by the unweighted pair group method with arithmetic averages (UPGMA), using GELCOMP version 4.2 (Applied Maths). As with many other *Campylobacter* species, a prominent protein band with variable position (36.1–43.2 kDa) was present in the profiles of the rabbit isolates (Fig. 2) and, for numerical analysis, the region was excluded to increase species discrimination (Vandamme *et al.*, 1991). Excluding this variable dense band region from the numerical analysis to enhance species-level discrimination resulted in a clear grouping of the rabbit isolates.

DNA–DNA hybridizations were subsequently performed between strains 150B^T and 116/07. For this purpose, DNA was extracted from 0.25–0.5 g (wet weight) cells as

described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed at 30 °C with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989) using an HTS7000 Bio Assay Reader (PerkinElmer) for the fluorescence measurements. A DNA–DNA hybridization value of 92 % was calculated.

The physiological characters of the novel species, determined using standard methods (On & Holmes, 1991a, b, 1992; Ursing *et al.*, 1994; On *et al.*, 1996), along with those of all *Campylobacter* reference strains are represented in Table 1 and in the species description. These characteristics allowed differentiation of the rabbit isolates from recognized *Campylobacter* species (Table 1).

Morphological characteristics were determined using transmission electron microscopy (TEM). For TEM, 48-h-old cells were negatively stained with 1 % (w/v) phosphotungstic acid (Sigma) and examined using a Zeiss E900 TEM microscope. Cells were spiral shaped, with bipolar unsheathed flagella; periplasmic fibres on the surface were not observed (data not shown).

For the determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8

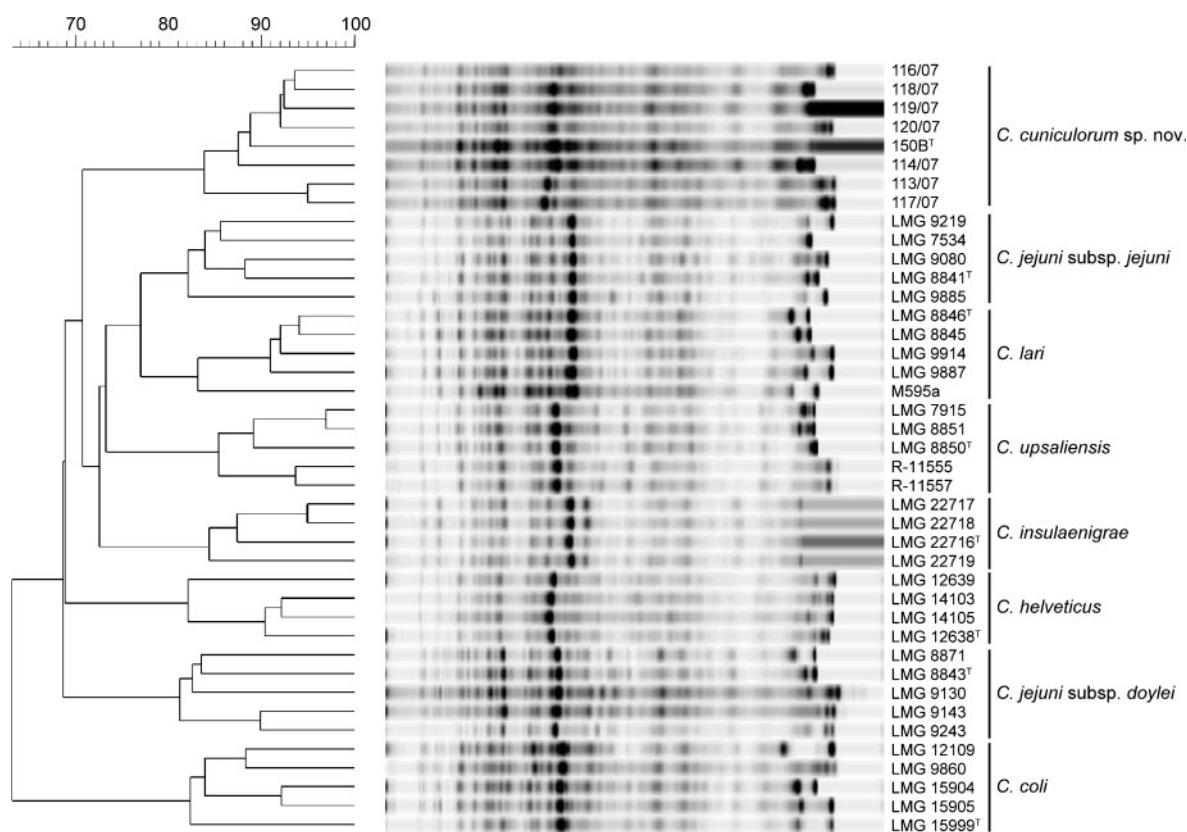


Fig. 2. Dendrogram of the eight strains of *Campylobacter cuniculorum* sp. nov. and representatives of other *Campylobacter* species based on UPGMA cluster analysis of one-dimensional SDS-PAGE cell protein profiles.

Table 1. Phenotypic characteristics of *Campylobacter* species

Species/subspecies: 1, *Campylobacter cuniculorum* sp. nov.; 2, *C. canadensis*; 3, *C. coli*; 4, *C. concisus*; 5, *C. curvus*; 6, *C. fetus* subsp. *fetus*; 7, *C. fetus* subsp. *venerealis*; 8, *C. gracilis*; 9, *C. helveticus*; 10, *C. hominis*; 11, *C. hyointestinalis* subsp. *hyointestinalis*; 12, *C. hyointestinalis* subsp. *lawsonii*; 13, *C. insulaenigrae*; 14, *C. jejuni* subsp. *doylei*; 15, *C. jejuni* subsp. *jejuni*; 16, *C. lanienae*; 17, *C. lari*; 18, *C. mucosalis*; 19, *C. rectus*; 20, *C. showae*; 21, *C. sputorum*; 22, *C. upsaliensis*. Data for reference species were taken from On *et al.* (1996), Foster *et al.* (2004), Vandamme *et al.* (2005) and Inglis *et al.* (2007). No taxa grow aerobically at 37 °C. +, 90–100 % Strains positive; (+), 75–89 % positive; v, 26–74 % positive; (–), 11–25 % positive; –, 0–10 % positive; ND, no data available. mCCDA, modified-charcoal cefoperazone deoxycholate agar; TSI agar, triple sugar-iron agar; TTC, triphenyl tetrazolium chloride.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
α-Haemolysis	+	–	(–)	(–)	(–)	–	v	–	+	ND	v	v	ND	+	+	+	v	–	+	+	+	+
Oxidase	+	+	+	v	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	v	+	+
Catalase	+	v	+	–	–	+	(+)	v	–	–	+	+	+	v	+	+	+	–	(–)	+	v	–
Alkaline phosphatase	–	–	–	v	v	–	–	–	–	–	–	(–)	ND	–	–	+	–	(+)	–	–	–	–
γ-Glutamyltranspeptidase	–	(+)	–	–	ND	–	ND	ND	–	ND	–	–	ND	–	–	ND	–	ND	ND	ND	–	–
Urease	–	v	–	–	–	–	–	–	–	–	–	–	–	–	–	–	v	–	–	–	v*	–
Hydrolysis of:																						
Hippurate	–	–	–	–	(–)	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
Indoxyl acetate	+	–	+	–	v	–	–	v	+	–	–	–	–	+	+	–	–	–	+	–	–	+
Reduction of:																						
Nitrate	+	v	+	(–)	+	+	+	(+)	+	–	+	+	+	–	+	+	+	–	+	+	+	+
Selenite	–	ND	v	(–)	–	(+)	–	–	–	–	+	+	ND	–	+	+	+	–	+	+	+	+
TTC	v	ND	+	–	v	–	–	–	–	ND	–	–	ND	v	+	ND	+	–	–	–	–	v
Trace H ₂ S production on TSI agar	–	v	–	–	(–)	–	–	–	–	–	+	+	–	–	–	–	–	+	–	v	+	–
Growth at/in/on:																						
25 °C (microaerobic)	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
37 °C (microaerobic)	+	+	+	+	v	+	+	–	+	+	+	+	+	+	+	+	+	+	–	v	+	+
42 °C (microaerobic)	(+)	+	+	(+)	v	(+)	–	v	+	(–)	+	+	–	–	+	+	+	+	(–)	v	+	+
37 °C (anaerobic)	–	+	–	+	+	(–)	v	+	–	+	–	+	–	–	–	+	–	+	+	+	+	–
Nutrient agar	+	–	+	(–)	+	+	+	+	(+)	ND	+	+	ND	+	+	ND	+	+	(–)	v	+	+
mCCDA	(+)	+	+	(–)	(+)	+	+	v	+	ND	+	+	ND	+	+	ND	+	+	–	+	(+)	+
MacConkey agar	–	+	v	–	(+)	(+)	v	(+)	–	–	v	v	ND	–	–	+	–	(+)	–	+	v	–
1 % Glycine	–	v	+	(–)	+	+	–	+	v	+	+	v	+	(–)	+	–	+	v	+	v	+	+
2 % NaCl	–	ND	–	(–)	v	–	–	v	–	+	–	–	–	–	–	–	(+)	+	v	+	+	–
1 % Bile	v	ND	(+)	–	–	+	+	–	+	ND	+	(+)	ND	+	+	ND	+	+	–	–	v	+
Requirement for H ₂	–	–	–	+	+	–	–	+	–	+	v	v	ND	–	–	–	–	+	+	+	–	–
Resistance to:																						
Cephalotin	(+)	–	+	–	–	–	–	–	–	–	(–)	–	+	–	+	+	+	–	–	–	–	(–)
Nalidixic acid	v	v	–	(+)	+	+	v	v	–	v	+	+	+	–	–	+	v	(+)	(+)	–	(+)	–

*Strains of biovar paraureolyticus are urease-positive; other strains are urease-negative (On *et al.*, 1998).

column maintained at 37 °C. The solvent was 0.02 M (NH₄)H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated λ -phage DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain 150B^T was 32.4 mol%. This value is within the range reported for the genus *Campylobacter* (29–47 mol%) (Vandamme *et al.*, 2005).

In conclusion, the results of this polyphasic taxonomic study indicate that the isolates recovered from the caecal contents of rabbits represent a homogeneous novel species within the genus *Campylobacter*, for which we propose the name *Campylobacter cuniculorum* sp. nov.

Description of *Campylobacter cuniculorum* sp. nov.

Campylobacter cuniculorum (cu.ni.cu.lo'rum. L. gen. pl. n. *cuniculorum* of rabbits).

Cells are spiral, Gram-negative rods, motile, 0.2–0.4 μ m wide and 1.9–3.3 μ m long, possessing a single flagellum at both poles. After subculturing on nutrient sheep-blood agar, colonies are grey-green, flat with rough margins and slightly mucoid-looking; after 72–96 h at 37 °C under microaerobic conditions, colonies are smooth, α -haemolytic, 1–2 mm in diameter. Colony appearance on modified-charcoal cefoperazone deoxycholate agar (mCCDA) and cefoperazone, amphotericin B, teicoplanin selective supplement (CAT) is similar to that on nutrient agar but growth on the first medium is slightly restricted. Strictly microaerobic. Able to grow at 37 °C and most strains grow at 42 °C; no growth at 25 °C or under anaerobic or aerobic conditions. Hydrogen is not required for growth. Oxidase and catalase are produced, but not urease, γ -glutamyltranspeptidase or alkaline phosphatase. Hydrolyses indoxyl acetate but not hippurate, and reduces nitrate but not selenite. Some strains reduce triphenyl tetrazolium chloride (TTC) and grow on nutrient agar without blood but not on MacConkey agar. No growth occurs in the presence of 1% (w/v) glycine and 2% (w/v) NaCl and only few strains grow in the presence of 1% (w/v) bile. Most strains are resistant to (μ g per disc) nalidixic acid (30) and cephalothin (30) by disc diffusion test. Strains have been recovered from rabbit caecal contents but pathogenicity is unknown. The G+C content of the type strain is 32.4 mol%.

The type strain is 150B^T (=LMG 24588^T =CCUG 56289^T), which was isolated from a rabbit in 2005.

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