Campylobacter subantarcticus sp. nov., isolated from birds in the sub-Antarctic region

Lies Debruyne,[1] Tina Broman,[2] Sven Bergström,[3] Björn Olsen,[4,5] Stephen L. W. On[6] and Peter Vandamme[1]

Correspondence
Lies Debruyne
Lies.Debruyne@UGent.be

[1]Department of Biochemistry, Physiology and Microbiology, Faculty of Sciences, Ghent University, Ledeganckstraat 35, B-9000 Gent, Belgium

[2]CBRN Defence and Security, FOI, Swedish Defence Research Institute, SE-901 82 Umeå, Sweden

[3]Department of Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden

[4]Department of Medical Sciences, Section of Infectious Diseases, Uppsala University, SE-751 85 Uppsala, Sweden

[5]Section for Zoonotic Ecology and Epidemiology, Department of Natural Sciences, Kalmar University, SE-391 82 Kalmar, Sweden

[6]Institute of Environmental Research and Science, Food Safety Programme, PO Box 29 181, Christchurch, New Zealand

Six Gram-stain-negative, spiral-shaped, microaerobic isolates were obtained during a sampling from wild birds in the sub-Antarctic region. Based on initial observations, these isolates were classified as *Campylobacter lari*-like. The isolates were further characterized by whole-cell protein and amplified fragment length polymorphism (AFLP) analysis, which revealed that they were distinct from *C. lari* and all other known species of the genus *Campylobacter*. Here, we present comprehensive phylogenetic, genomic and phenotypic evidence that these isolates represent a novel species within the genus *Campylobacter*, for which the name *Campylobacter subantarcticus* sp. nov. is proposed. The type strain is R-3023^T (=LMG 24377^T =CCUG 38513^T).

At the time of writing, the genus *Campylobacter* (Sebald & Veron, 1963) comprises 20 species and 8 subspecies with validly published names, with species found in both man and a wide range of domestic and wild animals and birds. Species most often associated with captive or free-living wild birds, either asymptomatic or with disease symptoms, include *Campylobacter lari* subsp. *lari* and *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, and urease-positive thermophilic *Campylobacter* (UPTC) *lari* isolates (Waldenström *et al.*, 2002, 2007). The more recently descibed species *Campylobacter canadensis* has been isolated exclusively from captive whooping cranes (Inglis *et al.*, 2007). The presence of zoonotic species in wild birds may provide a reservoir for human-pathogenic species, either through direct contact or through contamination of the environment.

During a sampling of wild birds and fur seals at Bird Island (54° 00′ S 38° 02′ W) in the South Georgian archipelago in 1996, a collection of *Campylobacter* isolates was obtained. Several of these isolates were initially designated *C. lari*-like, based on biochemical similarities. Six of these isolates were included in the present polyphasic taxonomic study: three were isolated from grey-headed albatrosses (*Diomedea chrysostoma*), two from black-browed albatrosses (*Diomedea melanophris*) and one from a gentoo penguin (*Pygoscelis papua*). No isolates could be obtained from Antarctic fur seals, suggesting that this species is restricted to birds. Strains were examined by whole-cell protein SDS-PAGE, amplified fragment length polymorphism (AFLP) and 16S rRNA and *hsp60* gene sequencing. Phenotypic characteristics were determined, and relevant DNA–DNA hybridizations were performed.

In February/March 1996, faecal swabs were taken from 10 adult female and 40 female Antarctic fur seal pups (*Arctocephalus gazella*), 30 adult gentoo penguins, 50 macaroni penguin chicks (*Eudyptes chrysolophus*), 50 black-

Abbreviation: AFLP, amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of LMG 24377^T, LMG 24374, LMG 24375 and LMG 24378 are AM933371, AM933372, AM933373 and AM933374, respectively. The GenBank/EMBL/DDBJ accession numbers for the *hsp60* gene sequences of LMG 24377^T and LMG 24374 are AM933375 and AM933376, respectively.

An UPGMA dendrogram of partial whole-cell protein SDS-PAGE profiles is available with the online version of this paper.

browed albatross chicks and 50 grey-headed albatross chicks. Faecal samples were collected using cotton wool swabs inserted into the rectum or cloaca. Samples were stored in a charcoal transport medium (Transwab, BioDisc, Solna, Sweden) at 5–10 °C, transported to Sweden and cultured within three weeks. Samples were plated on *Campylobacter* selective medium [42.5 g Columbia agar base l^{-1} (Becton Dickinson), 5 % citrated horse blood, 10 mg vancomycin l^{-1}, 2500 IE polymyxin B l^{-1}, 5 mg trimethoprim l^{-1}] and incubated for 48 h at 42 °C under microaerobic conditions. Colonies showing a Gram-stain-negative seagull-like cell morphology under light microscopy were subcultured onto Mueller-Hinton agar plates supplemented with 5% horse blood. Samples were stored at −80 °C in trypticase soy broth supplemented with 15 % glycerol.

Strains were cultured on Mueller–Hinton agar supplemented with 5 % horse blood at 37 °C for 48 h under microaerobic conditions (approx. 4 % O_2, 6.5 % CO_2, 6.5 % H_2, 83 % N_2). DNA was extracted as described by Pitcher *et al.* (1989).

Protein extraction and SDS-PAGE were performed as described by Pot *et al.* (1994). For whole-cell protein SDS-PAGE analysis, similarity of the normalized SDS-PAGE patterns obtained was determined by the Pearson product moment correlation coefficient, after which clustering was performed by UPGMA, using BioNumerics version 4.61 (Applied Maths, Belgium). For numerical analysis, a variable dense band region (36.1–43.2 kDa) (Vandamme *et al.*, 1990) was excluded to increase species discrimination. The results of the numerical analysis, in combination with visual inspection of the SDS-PAGE patterns, demonstrated that the SDS-PAGE patterns of the novel species were distinct from those of *C. lari*, and all other known species of the genus *Campylobacter* (Supplementary Fig S1, available in IJSEM Online).

AFLP analysis was performed as described by Debruyne *et al.* (2009). After normalization, the AFLP profiles obtained were included in an in-house AFLP reference database, containing profiles from type and reference strains of all established species of the genus *Campylobacter*. The similarity between profiles was determined by the Pearson correlation coefficient, and cluster analysis was performed by UPGMA, using BioNumerics v 4.61. AFLP profiles from the six strains representing the novel species were divergent from those of strains of other species of the genus *Campylobacter*, and formed a distinct cluster (Fig. 1).

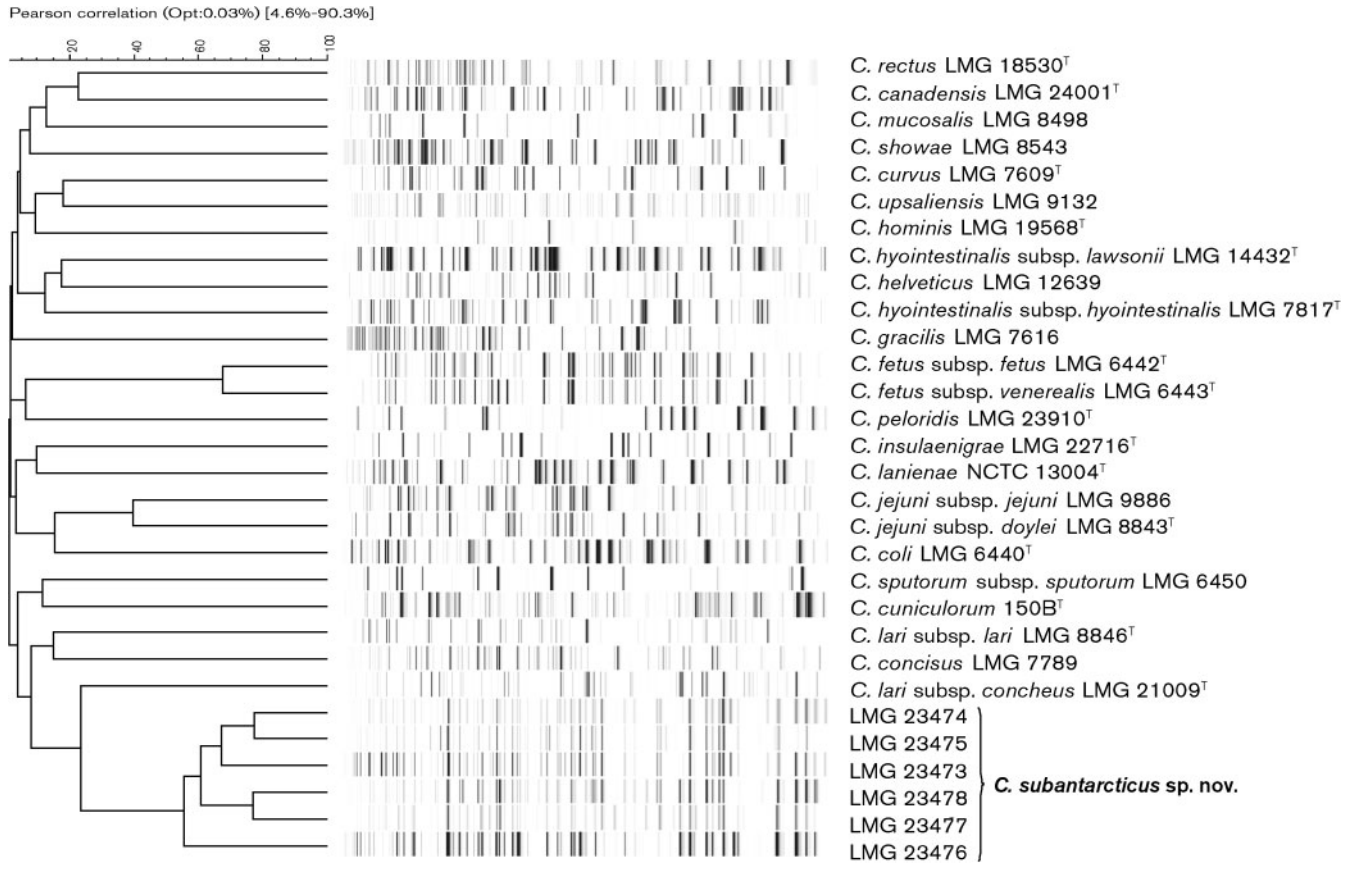

Fig. 1. Dendrogram representing the AFLP fingerprints of six strains representing the novel species *Campylobacter subantarcticus* sp. nov. and selected *Campylobacter* reference strains. Similarity was determined by the Pearson product moment correlation coefficient and clustering was performed by UPGMA.

To support the delineation of the groups defined by the above genomic and proteomic analyses, phenotypic testing was performed according to the phenotypic identification scheme described by On *et al.* (1996), including 67 phenotypic characteristics. Methods for biochemical testing were as described previously (On & Holmes, 1991a, b, 1992). The novel species belongs to the thermotolerant *Campylobacter* group, can grow in microaerobic conditions, and requires no hydrogen for growth. Differentiating characteristics are listed in Table 1, and a full phenotype description is given below.

To determine the phylogenetic position of the novel species, 16S rRNA gene sequences of strains LMG 24374, LMG 24375, LMG 24377^T and LMG 24378 (randomly selected) were determined as described previously (Vandamme *et al.*, 2006). Sequences were assembled using BioNumerics v 5.1. Comparison by the FASTA algorithm to the EMBL sequence database revealed that the nearest phylogenetic neighbours were *Campylobacter lari* subsp. *concheus*, *C. lari* subsp. *lari*, *C. jejuni*, *C. coli*, *C. insulaenigrae* and *C. peloridis*, all with similarity levels exceeding 97 %. Strains LMG 24375, LMG 24377^T and LMG 24378 had identical 16S rRNA gene sequences (100 % sequence similarity), while LMG 24374 was slightly more divergent (99.5 %). Sequences were aligned using the CLUSTAL_X software package (Thompson *et al.*, 1997), and clustering was performed by the neighbour-joining method (Saitou & Nei, 1987) using BioNumerics v 5.1. Unknown bases were discarded for the analysis. Bootstrap values were determined using 500 replicates (Fig. 2). Polymorphisms within the 16S rRNA gene were inadequate to distinguish among the novel taxon and *C. lari* subsp. *concheus*, with interspecies sequence similarities (99.4–99.9 %) being equal to or exceeding intraspecies sequence similarities (99.5–100 %). To improve species discrimination, partial *hsp60* gene sequences of LMG 24374 and LMG 24377^T were determined as described previously (Debruyne *et al.*, 2009). Kärenlampi *et al.* (2004) demonstrated that phylogeny based on the *hsp60* gene sequence, coding for the 60 kDa heat-shock protein, was similar to that of the 16S rRNA gene. However, *hsp60* was found to provide a better resolution for species of the genus *Campylobacter*, with lower interspecies sequence similarities and high intraspecies sequence similarities. Pairwise comparison of *hsp60* gene sequences from the novel taxon and from *C. lari* subsp. *concheus* demonstrated a clear separation between intraspecies (100 %) and interspecies (93.3–93.9 %) sequence similarities, making species discrimination feasible (Fig. 3).

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 column maintained at 37 °C. The solvent was 0.02 M $(NH_4)H_2PO_4$ (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 LMG 24377^T was 30 %, which falls within the range reported for members of the genus *Campylobacter*, i.e. 29–47 %.

Table 1. Phenotypic characteristics differentiating the novel strains from species of the genus *Campylobacter*.

Species: 1, *Campylobacter subantarcticus* sp. nov. (n=6); 2, *C. canadensis*; 3, *C. coli*; 4, *C. concisus*; 5, *C. cuniculorum*; 6, *C. curvus*; 7, *C. fetus* subsp. *fetus*; 8, *C. fetus* subsp. *venerealis*; 9, *C. gracilis*; 10, *C. helveticus*; 11, *C. hyointestinalis*; 12, *C. hominis*; 13, *C. insulaenigrae*; 14, *C. jejuni*; 15, *C. lanienae*; 16, *C. lari* subsp. *concheus*; 17, *C. lari* subsp. *lari*; 18, *C. mucosalis*; 19, *C. peloridis*; 20, *C. rectus*; 21, *C. showae*; 22, *C. sputorum*; 23, *C. upsaliensis*. +, All strains positive; −, all strains negative; (+), 80–94 % strains positive; (−), 5–33 % strains positive; V, 35–67 % strains positive; ND, data not available. Additional data for reference species were taken from Inglis *et al.* (2007), Lawson *et al.* (2001), On *et al.* (1996) and Zanoni *et al.* (2009).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Catalase activity	+	V	+	−	+	−	+	(+)	(−)	−	+	−	+	(+)	+	+	+	−	+	(−)	+	V	+
Hippurate hydrolysis	−	−	−	−	−	(−)	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
H_2S production (TSI)	−	V	−	(−)	−	(−)	−	−	−	−	−	(+)	−	−	−	ND	−	+	ND	−	V	+	−
Growth at 42 °C	+	+	+	(+)	(+)	V	(+)	−	(−)	+	+	V	−	V	+	+	+	+	+	(−)	V	(+)	+
Requirement for H_2	−	−	−	+	−	+	−	−	+	−	V	+	ND	−	−	−	−	+	−	+	+	−	−
Alpha-haemolysis	+	−	(−)	(−)	+	(−)	−	(−)	−	+	V	−	+	(+)	+	ND	+	(−)	ND	+	+	+	+
Growth on:																							
MacConkey agar	(−)	−	V	−	−	(+)	(+)	V	(+)	−	V	−	−	(−)	+	+	(+)	(+)	+	−	+	V	−
Nutrient agar	−	−	+	(−)	+	+	+	(+)	+	(+)	+	+	V	+	−	+	+	+	+	(−)	V	(+)	+
Glycine (1 %)	(+)	V	(+)	(−)	−	+	+	(−)	+	V	V	+	−	V	−	+	+	V	+	+	V	+	+
Safranin (0.02 %)	−	ND	+	(−)	ND	+	+	(+)	+	−	+	−	−	V	−	−	+	+	−	−	−	(+)	+
Sodium deoxycholate (0.1 %)	−	ND	+	(−)	ND	(+)	+	(+)	(+)	(−)	V	−	+	V	−	V	+	−	V	−	−	V	V
Nalidixic acid (32 mg l^{-1})	+	V	−	(+)	V	+	+	V	V	−	+	(+)	+	−	+	−	(+)	(+)	(+)	(+)	−	(+)	−
Cephalothin (32 mg l^{-1})	−	−	+	−	(+)	−	−	−	−	−	(−)	−	+	V	+	+	+	V	(−)	−	−	−	(−)
Metrodinazole (4 mg l^{-1})	(−)	ND	(+)	(−)	ND	−	(+)	V	−	V	V	−	+	V	+	+	+	(+)	+	−	+	(−)	(+)
Carbenicillin (32 mg l^{-1})	−	ND	(+)	−	ND	−	−	−	−	V	−	−	+	V	+	+	+	−	−	−	−	−	−

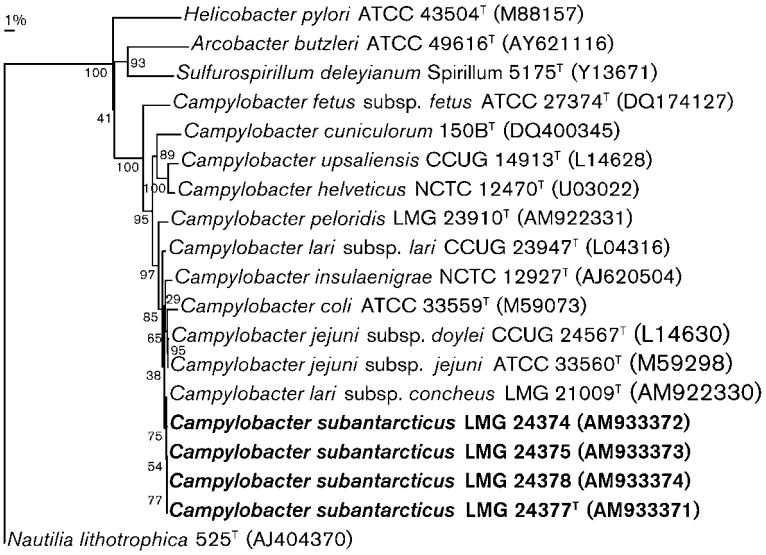

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences. Bootstrap values (%) are indicated at the nodes. Bar, 1% sequence divergence.

DNA–DNA hybridizations were performed between strain LMG 24377^T and type strains of its closest relatives, i.e. *C. lari* subsp. *lari*, *C. lari* subsp. *concheus*, *C. peloridis*, *C. jejuni* subsp. *jejuni*, *C. coli* and *C. insulaenigrae*. DNA was extracted from 0.25–0.5 g (wet wt) cells as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C. Reciprocal experiments were performed for every pair of strains and standard deviation values ranged from 1–8 %. DNA–DNA hybridization values between strain LMG 24377^T and the type strains of *C. lari* subsp. *lari* (LMG 8846^T), *C. lari* subsp. *concheus* (LMG 21009^T), *C. peloridis* (LMG 23910^T), *C. jejuni* subsp. *jejuni* (LMG 8841^T), *C. coli* (LMG 6440^T), and *C. insulaenigrae* (LMG 22716^T) were 57, 55, 38, 21, 16 and 41 %, respectively. All of these values are well below the threshold of 70 % for species delineation (Stackebrandt & Goebel, 1994).

The present study demonstrates that the six bird isolates represent a novel species within the genus *Campylobacter* which can be distinguished from other species of this genus by whole-cell protein electrophoresis, AFLP fingerprinting, *hsp60* gene sequence analysis and biochemical characteristics. We formally propose to classify these strains as *Campylobacter subantarcticus* sp. nov., with LMG 24377^T as the type strain.

Description of *Campylobacter subantarcticus* sp. nov.

Campylobacter subantarcticus (sub.ant.arc′ti.cus N.L. masc. adj. *subantarcticus* pertaining to the sub-Antarctic region, from where the organism was first isolated).

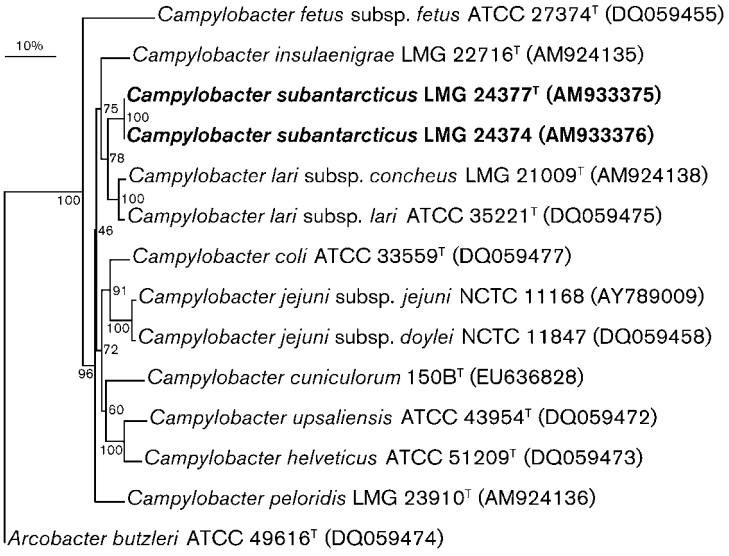

Fig. 3. Neighbour-joining tree based on partial *hsp60* gene sequences. All sequences are 555 bp in length, with the exception of the sequence for *C. cuniculorum*, which is 489 bp in length. Bootstrap values (%) are indicated at the nodes. Bar, 10% sequence divergence.

Cells are slightly curved, Gram-stain-negative rods. Colonies are colourless, round, entire, convex and 1–1.5 mm in diameter after culture on 5 % blood agar for 72 h under microaerobic conditions. Single polar flagella are observed. Oxidase and catalase-positive. Does not hydrolyse hippurate or indoxyl acetate, and does not produce H_2S on TSI agar. Selenite is not reduced. Grows at 37 °C and 42 °C, but not at room temperature (18–22 °C) or 25 °C, under microaerobic conditions. No growth observed under aerobic conditions at either 37 or 25 °C. Growth observed at 37 °C under anaerobic conditions on unsupplemented blood agar and on blood agar supplemented with 0.1 % trimethylamine *N*-oxide. Grows on media containing 1.0–2.0 % desiccated ox-bile, 2.0 % NaCl, 32 mg nalidixic acid ml^{-1}, 100U 5-fluorouracil ml^{-1} and 0.05 % sodium fluoride. Most strains grow on media containing 1 % glycine. Most strains do not grow on media containing 4 mg metronidazole ml^{-1} or on MacConkey agar. No growth observed on unsupplemented nutrient agar, casein agar, lecithin agar, tyrosine agar, or on media containing 0.02 or 0.05 % safranin, 0.1 % potassium permanganate, 0.005 % crystal violet, 3.5–4.0 % NaCl, 0.1 % sodium deoxycholate, 32 mg cephalothin ml^{-1} or 32 mg carbenicillin ml^{-1}. Alpha-haemolysis observed on 5 % blood agar

Pathogenicity unknown. Strains have been recovered from wild birds in the sub-Antarctic region.

The type strain is R-3023^T (=LMG 24377^T =CCUG 38513^T), which was isolated from a grey-headed albatross in 1996.

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