Novel *Campylobacter lari*-like bacteria from humans and molluscs: description of *Campylobacter peloridis* sp. nov., *Campylobacter lari* subsp. *concheus* subsp. nov. and *Campylobacter lari* subsp. *lari* subsp. nov.

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A polyphasic study was undertaken to clarify the taxonomic position of *Campylobacter lari*-like strains isolated from shellfish and humans. The diversity within the strain collection was initially screened by means of fluorescent amplified fragment length polymorphism analysis and whole-cell protein electrophoresis, revealing the existence of two clusters distinct from *C. lari* and other *Campylobacter* species. The divergence of these clusters was confirmed by phenotypic analysis and by 16S rRNA and *hsp60* gene sequence analysis. Phylogenetic analysis identified *C. lari*, *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter insulaenigrae* as the closest phylogenetic neighbours of both taxa. DNA–DNA hybridizations revealed that one cluster, comprising 10 strains, represented a novel *Campylobacter* species, for which the name *Campylobacter peloridis* sp. nov. is proposed, with 2314BVA T (=LMG 23910 T =CCUG 55787 T) as the type strain. The second cluster, comprising six strains, represents a novel subspecies within the species *C. lari*, for which the name *Campylobacter lari* subsp. *concheus* subsp. nov. is proposed, with 2897R T (=LMG 21009 T =CCUG 55786 T) as the type strain. The description of *C. lari* subsp. *concheus* has the effect of automatically creating the subspecies *Campylobacter lari* subsp. *lari* subsp. nov. (type strain LMG 8846 T =NCTC 11352 T).

The genus *Campylobacter* was created by Sebald & Veron (1963) and has since been extended with species originating from both humans and animals. At the time of writing, the genus comprises 18 species and 6 subspecies with validly published names. *Campylobacter lari* was described by Benjamin et al. (1983) and has been isolated from the intestinal contents of seagulls and animals, from river water and from shellfish. In humans, strains have been isolated sporadically from diarrhoeic faeces and from cases of bacteraemia and other extraintestinal infections, in both immunocompetent and immunodeficient patients. The species was originally referred to as the nalidixic-acid-resistant thermophilic *Campylobacter* (NARTC) group, but the existence of biochemical variants has been reported, including urease-producing (so-called UPTC variants), nalidixic-acid-susceptible (NASC) and urease-producing, nalidixic-acid-susceptible (UP-NASC) strains. Endtz et al. (1997) observed a striking heterogeneity amongst and within the different groups of *C. lari* variants; this was confirmed by Duim et al. (2004), who identified four distinct subgroups using numerical analysis of amplified fragment length polymorphism (AFLP) profiles and of partial protein profiles.

In a long-term study of the diversity of *Campylobacter*-like organisms, we collected *C. lari*-like strains from a wide range of isolation sources and geographical regions. Strain details are provided in Supplementary Table S1, available in IJSEM Online. Our aim was to study the aforementioned diversity and re-evaluate the taxonomic position of these strains, using a polyphasic approach.
Diversity within the strain collection was initially assessed using whole-cell protein electrophoresis and AFLP analysis. Selected isolates from the observed clusters were further investigated by means of 16S rRNA and hsp60 gene sequence analysis and by phenotypic analysis. Finally, DNA–DNA hybridizations were performed between representatives of each novel group and closely related taxa.

Strains were grown on Mueller–Hinton agar, supplemented as necessary with 5% sterile horse blood, at 37 °C for 24–48 h, after which DNA was extracted as described by Pitcher et al. (1989).

AFLP analysis was performed as described by Duim et al. (1999). In brief, 1 µg genomic DNA was digested with the HindIII–HhaI restriction enzyme combination. After digestion, site-specific adaptors were ligated to the restriction fragments. Primers complementary to the adaptor and restriction site sequence were used in subsequent pre-selective and selective PCR amplification reactions. The amplified and fluorescently labelled fragments were loaded on a denaturing polyacrylamide gel on an ABI Prism 377 automated sequencer (Applied Biosystems). GeneScan 3.1 (Applied Biosystems) was used for data collection, and the profiles generated were imported, using the CrvConv filter, in BioNumerics 4.61 (Applied Maths) for normalization and further analysis. Similarity between normalized profiles was determined using Pearson’s product–moment correlation coefficient and a dendrogram was constructed using the unweighted pair group method with arithmetic means (UPGMA) algorithm. Numerical analysis differentiated two groups of C. lari-like organisms distinct from the classical C. lari and C. lari UPTC clusters, with different clusters exhibiting less than 35% similarity. The C. lari UPTC reference strains were selected from previous studies (Duim et al., 2004; On & Harrington, 2000; Vandamme et al., 1991) and formed two distinct clusters, confirming previous reports on the extensive genetic diversity within this group (Matsuda et al., 2003; On & Harrington, 2000). The taxonomy of the C. lari UPTC strains is the subject of a separate study (C. Fitzgerald, L. O. Helsel, A. Steigerwalt, P. Vandamme, J. Pruckler, M. Daneshvar, M. A. Nicholson, C. S. Harrington, P. I. Fields and S. L. W. On, unpublished results). Reference strains from Campylobacter jejuni subsp. jejuni, C. jejuni subsp. doylei, Campylobacter coli and Campylobacter insulaenigrae, the closest phylogenetic neighbours of C. lari on the basis of 16S rRNA gene sequence analysis, could be differentiated from all C. lari-like strains examined (Fig. 1). Clusters I (n=10) and II (n=6) correspond to genogroups IV and III, respectively, as described by Duim et al. (2004).

For whole-cell protein SDS-PAGE, isolates were grown on Mueller–Hinton agar supplemented with 5% sterile horse blood and incubated at 37 °C for 48 h under microaerobic conditions (O2/CO2/H2/N2, approx. 4:6.5:6.5:83). The
preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot et al. (1994). Normalization of densitometric traces was performed using GeCompass 4.2 (Applied Maths). Numerical analysis was performed using Pearson’s product–moment correlation coefficient and UPGMA with BioNumerics 4.61 software. For numerical analysis, only the region comprising approx. 20–35 kDa was used, as this part showed additional heterogeneity, which was also described by Duim et al. (2004). Cluster I, as observed in the AFLP analysis, was also observed in the protein analysis and was divergent from the classical C. lari cluster (83 % similarity). Cluster II could also be discriminated from the classical C. lari cluster, but at a higher similarity level (88 %) (Fig. 2). The C. lari-like strains could also be differentiated from C. jejuni, C. coli and C. insulaenigrae reference strains (data not shown).

To support the delineation of the groups defined by the above genomic and proteomic analyses, phenotypic testing was performed using tests known to reveal variation among C. lari strains (On et al., 1996; S. W. L. On, unpublished results). These included evaluation of growth on media containing 2.0 % NaCl, 1.0 % glycine, 0.05 % safranin, nalidixic acid (32 mg l⁻¹), cefalothin (32 mg l⁻¹), metronidazole (4 mg l⁻¹), carbenicillin (32 mg l⁻¹) and 0.1 % sodium deoxycholate. Growth on unsupplemented Campylobacter charcoal deoxycholate agar, growth on unsupplemented nutrient agar (no. 2; Oxoid) and the presence of urease activity were also evaluated. The methods used for biochemical testing were as described previously (On & Holmes, 1991a, b, 1992). Characteristics that served to differentiate clusters I and II from each other and from other Campylobacter species are listed in Table 1.

The phylogenetic positions of a selection of isolates from clusters I (LMG 23910T and LMG 11251) and II (LMG 21009T and LMG 11760) were determined by analysing almost-complete 16S rRNA gene sequences. Amplification, purification and sequencing of the 16S rRNA genes were performed as described by Vandamme et al. (2006). Sequence assembly was performed using BioNumerics 4.61 and selected sequences were aligned using CLUSTAL_X. Subsequently, the aligned sequences were imported into BioNumerics for phylogenetic analyses and bootstrap analysis (500 replicates). Ambiguous bases were discarded for the analysis and a rooted phylogenetic tree was constructed using the neighbour-joining method, with Caminibacter hydrogeniphilus AM1116T as an outgroup (Fig. 3). The tree mainly comprises the closely related, thermotolerant Campylobacter species; a full phylogenetic tree for the genus Campylobacter was published recently (Debruyne et al., 2008). The 16S rRNA gene sequence similarity between strains LMG 23910T and LMG 11251 was 100 %, while that between strains LMG 21009T and LMG 11760 was 99.9 %. The similarity between the 16S rRNA gene sequences of LMG 23910T and LMG 21009T was 97.6 %. Comparison with sequences available in the EMBL database indicated that, for both clusters, the closest phylogenetic neighbours (showing >97 % sequence similarity) included C. lari, C. jejuni, C. coli and C. insulaenigrae.

Fig. 2. UPGMA dendrogram of partial whole-cell protein SDS-PAGE profiles. The molecular mass markers used were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.2 kDa).
Table 1. Differential phenotypic characteristics of the novel strains and related Campylobacter species

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Catalase activity | + | + | + | V | + | − | − | + | + | (+) | (−) | − | − | + | (+) | − | − | − | − | − | V | + |
| Hippurate hydrolysis | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Growth at/with/in: | | | | | | | | | | | | | | | | | | | | | | | |
| 42 °C | + | + | + | − | + | + | (+) | V | (+) | (−) | − | + | V | V | + | (−) | V | (+) | + | + | + | V | + |
| NaCl (2 %) | (−) | + | + | − | ND | − | (−) | V | − | − | (−) | (−) | − | V | − | − | (+) | V | + | + | − | (−) | V | + |
| Nutrient agar | + | + | V | − | + | (−) | + | (+) | + | (+) | + | + | + | − | + | (−) | V | (−) | (−) | V | + | + |
| Glycine (1 %) | + | + | − | V | (−) | + | (−) | + | + | V | V | V | V | − | V | V | V | + | + | − | (−) | V | + |
| Safranin (0.05 %) | − | − | − | ND | + | (−) | + | (+) | + | − | − | V | − | − | − | − | − | + | − | − | − | (−) | + |
| Sodium deoxycholate (0.1 %) | V | V | + | ND | + | (−) | (+) | (−) | (+) | (−) | (−) | V | V | V | − | V | V | − | − | V | V | V |
| Nalidixic acid (32 mg l⁻¹) | (−) | − | (+) | − | V | − | (−) | + | + | V | V | V | − | V | − | + | (+) | (+) | − | (−) | + | (−) | (−) |
| Cephalothin (32 mg l⁻¹) | (−) | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Metronidazole (4 mg l⁻¹) | + | + | + | ND | (+) | (−) | − | (−) | V | V | V | V | V | V | + | (−) | − | (+) | (−) | (−) | | |
| Carbenicillin (32 mg l⁻¹) | − | + | + | ND | (+) | − | − | − | − | V | − | V | − | V | V | − | V | − | − | − | − | − | − |

Phylogenetic relationships were further examined using sequencing of the hsp60 gene (also known as cpn60 or groEL). The hsp60 gene encodes the 60 kDa chaperonin protein that is found in virtually all members of the Bacteria; the usefulness of this target for phylogenetic analysis is well established (Kärenlampi et al., 2004). Kärenlampi et al. (2004) introduced phylogenetic analysis based on the hsp60 gene for the genus Campylobacter. By cloning and sequencing a PCR-amplified fragment, they obtained a phylogenetic tree with topology similar to that of 16S rRNA gene sequence-based trees, but with increased resolution due to lower interspecies similarities in the hsp60 gene. The use of the hsp60 gene as a phylogenetic marker for the genus Campylobacter was further optimized by the implementation of direct sequencing of PCR-amplified hsp60 gene sequences (Hill et al., 2006).

The reaction mixture and cycling conditions for the PCR were optimized in the present study. The PCR amplification reactions contained 1 × PCR buffer, 200 μM dNTPs,
C. lari

value between LMG 23910T and LMG 21009T was 51\%.

78, 23, 19 and 35\% respectively. The DNA–DNA binding between LMG 23910T (cluster I) and LMG 21009T (cluster II) were 29 and 89\%.

Hill et al., 2006). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 46 °C for 30 s and elongation at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. Sequencing and further analysis were performed as described for 16S rRNA gene sequencing. Comparison of partial hsp60 gene sequences (555 bp) with sequences in the EMBL database, using the FASTA algorithm, revealed that, for cluster I strains LMG 23910T and LMG 11251, the closest neighbour was C. lari (88–90\%). For cluster II strains LMG 21009T and LMG 11760, the closest phylogenetic neighbour was again C. lari, though higher similarity levels were observed (>96\%), followed by C. jejuni and C. coli (<89\%). A rooted neighbour-joining tree, with Arcobacter butzleri ATCC 49616T as an outgroup, representing the hsp60 gene sequence phylogeny, is presented in Supplementary Fig. S1.

For the determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated using HPLC with a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M \((\text{NH}_4)_2\text{HPO}_4\) (pH 4.0) with 1.5 % acetonitrile. Non-methylated \(\lambda\)-phage DNA (Sigma) was used as the calibration reference. The DNA G+C contents of strains LMG 23910T (cluster I) and LMG 21009T (cluster II) were 29 and 30 mol\%, respectively. These values are within the range (29–47 mol\%) reported for the genus Campylobacter.

For LMG 23910T, the values are well below the threshold (70\%) suggested for species delineation (Stackebrandt & Goebel, 1994), but the results for LMG 21009T and LMG 8846T indicate that these strains represent the same species. This confirms preliminary results from hybridization experiments performed between strain LMG 11760 (cluster II) and LMG 8846T (Duim et al., 2004). Both the genotypic and phenotypic analyses allowed us to distinguish these taxa from established Campylobacter species. Therefore we propose that the cluster I strains represent a novel species, for which we propose the name Campylobacter peloridis sp. nov., and that the cluster II strains represent a novel subspecies within C. lari, for which we propose the name Campylobacter lari subsp. conchae subsp. nov.

Emended description of Campylobacter lari

Benjamin et al. 1984

Cells are Gram-negative, spiral to curved rods, 0.3 x 1.7–2.4 \(\mu\)m in size. After 72 h culture under microaerobic conditions on 5 % blood agar, colonies are colourless, round, entire, convex and 1–1.5 mm in diameter. Cells show rapid darting motility by means of single bipolar flagella. Oxidase- and catalase-positive. Strains do not hydrolyse hippurate. Under microaerobic conditions, strains grow at 37 and 42 °C but not at 25 °C. No growth occurs in air at either 25 or 37 °C. Acid from glucose is not detected. Growth under microaerobic conditions is observed on unsupplemented Campylobacter charcoal deoxycholate agar and on blood agar media containing 1.0 % glycine, 2.0 % NaCl and 32 mg carbenicillin 1\(^{-1}\). Strains have been isolated from cases of human diarrhoea and bacteraemia, from horse intestine, from the faeces of wild birds, dogs and chickens, from environmental water samples and from shellfish.

Description of Campylobacter lari subsp. lari subsp. nov.

Campylobacter lari subsp. lari (la’ri. L. gen. n. lari of a gull).

Strains conform to the species description given above and also exhibit the following characteristics. Growth occurs on media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood media containing 0.1 % trimethylamine \(N\)-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. \(\alpha\)-Haemolysis is observed on 5 % blood.

For 5.0 U Taq polymerase, 5 pmol forward primer H729 (5'-CGCCAGGTTTCCGTCGAGCAIIIICGIGGIGGICGACCCTCGC-3'), 5 pmol reverse primer H730 (5'-AGCGGGATAACATTTCCACAGGAYKIYKTICTICRCRAAICCGGGIGGYTT-3') and 25 ng genomic DNA, with the final volume adjusted to 25 \(\mu\)l. The amplification primers include landing sites for the sequencing primers (underlined), enabling direct sequencing (Hill et al., 2006).

The DNA–DNA hybridization experiments performed between strain LMG 11760 (cluster II) and LMG 8846T (Duim et al., 2004). Both the genotypic and phenotypic analyses allowed us to distinguish these taxa from established Campylobacter species. Therefore we propose that the cluster I strains represent a novel species, for which we propose the name Campylobacter peloridis sp. nov., and that the cluster II strains represent a novel subspecies within C. lari, for which we propose the name Campylobacter lari subsp. conchae subsp. nov. With reference to data from previous studies (Benjamin et al., 1983; On et al., 1996), we correspondingly emend the species description of C. lari and propose Campylobacter lari subsp. lari subsp. nov. to encompass the classical nalidixic-acid-resistant, urease-negative isolates typified by the type strain. The divergence of the novel taxa presented here has been confirmed in previous studies, including a multilocus sequence typing system analysis (Miller et al., 2005) and a phylogenetic analysis of a partial GTPase-encoding gene (van Doorn et al., 1998).
Novel Campylobacter lari-like bacteria

agar. Microaerobic growth occurs on unsupplemented nutrient agar, on nutrient agar media containing 0.02 or 0.05% safranin and 0.1% sodium deoxycholate and on blood agar media containing 4 mg metronidazole l⁻¹, 32 mg carbenicillin l⁻¹ and 64 mg cefoperazone l⁻¹. Strains have been isolated from cases of human diarrhoea and bacteraemia, from horse intestine and from the faeces of wild birds, dogs and chickens.

The type strain, LMG 8846T (=NCTC 11352T), was isolated from gull faeces in 1976.

Description of Campylobacter lari subsp. concheus subsp. nov.

Campylobacter lari subsp. concheus (con’che.us. L. masc. adj. concheus of, or pertaining to, shellfish).

Strains conform to the species description given above. Strains of this subspecies can be distinguished from C. lari subsp. lari by their inability to grow on media containing 0.05% safranin. Growth on media containing 0.1% sodium deoxycholate is strain dependent. Pathogenicity unknown.

Strains have been isolated from human faeces and from shellfish. The type strain, 2897RT (=LMG 21009T =CCUG 55786T), was isolated from shellfish in 1993.

Description of Campylobacter peloridis sp. nov.

Campylobacter peloridis (pe’lo.ri.dis. L. gen. n. peloridis of a large shellfish, of the giant mussel).

Cells are slightly curved, Gram-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. Oxidase- and catalase-positive. Growth occurs on media containing 1.0% glycine and 4 mg metronidazole l⁻¹. Most known strains (8/10) grow on media containing 2% NaCl or 32 mg nalidixic acid l⁻¹. Most known strains (9/10) do not grow on media containing 32 mg cephalothin l⁻¹. Strains do not grow on media containing 0.05% safranin or 32 mg carbenicillin l⁻¹. Pathogenicity is unknown.

Strains have been recovered from human faeces, from dialysis fluid and from shellfish. The type strain, 2314BVAT (=LMG 23910T =CCUG 55787T), was isolated from shellfish in 1993.

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References


