Campylobacter insulaenigrae sp. nov., isolated from marine mammals

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Phenotypic and phylogenetic studies were performed on four *Campylobacter*-like organisms recovered from three seals and a porpoise. Comparative 16S rRNA gene sequencing studies demonstrated that the organisms represent a hitherto unknown subline within the genus *Campylobacter*, associated with a subcluster containing *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari*. DNA–DNA hybridization studies confirmed that the bacteria belonged to a single species, for which the name *Campylobacter insulaenigrae* sp. nov. is proposed. The type strain of *Campylobacter insulaenigrae* sp. nov. is NCTC 12927^T (=CCUG 48653^T).

In 1913, McFadyean & Stockman recorded the isolation of *Vibrio fetus* in pure culture and its association with abortion in sheep and cattle (McFadyean & Stockman, 1913). In 1963, this organism was transferred to the newly proposed genus *Campylobacter* (Sebald & Véron, 1963). During the 1970s, a renewed interest in *Campylobacter* followed the recognition of *Campylobacter jejuni* and *Campylobacter coli* as a cause of diarrhoea in humans (Cooper & Slee, 1971; Dekeyser *et al.*, 1972) and campylobacters soon became recognized as the commonest bacterial cause of enteritis (Griffiths & Park, 1990; Penner, 1988). In addition, the implementation of improved phylogenetic methods of analysis permitted closer taxonomic scrutiny of these organisms, resulting in several novel species, and led to the proposal of rRNA superfamily VI of the *Proteobacteria* as including the genera *Campylobacter*, *Helicobacter* and *Arcobacter* (Vandamme *et al.*, 1991). According to On (2001), the genus *Campylobacter* contains 16 species with a further six subspecies. In the course of bacteriological investigations of rectal swabs from free-ranging seals and also following post-mortem examination of seals and cetaceans, four *Campylobacter*-like organisms were recovered from three seals and a porpoise. In this paper, we describe the cultural and biochemical characteristics of these bacteria and the results of a polyphasic taxonomic investigation.

Three *Campylobacter*-like organisms were isolated from rectal swabs collected from three common seals (*Phoca vitulina*) during a capture–release programme (Thompson *et al.*, 1992). A fourth strain was recovered from a sample of small intestine taken from a harbour porpoise (*Phocoena phocoena*) carcass submitted under the Scottish Strandings Scheme. Isolation was made on Columbia blood agar base (Oxoid) supplemented with Blaser–Wang selective supplement (Oxoid) and 5% (w/v) citrated sheep blood agar (CSBA) (Oxoid). Plates were incubated at 37 °C in a microaerobic atmosphere achieved by jar evacuation and filling with a prepared gas mixture containing $N_2/CO_2/O_2$

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Abbreviation: SAFLP, single-enzyme amplified-fragment length polymorphism.

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(84 : 10 : 6). Culture plates were examined for growth after 2, 4 and 7 days incubation. Isolates were transferred to fresh citrated sheep blood agar plates for maintenance and characterization tests. The four strains were examined in a range of tests, the methods for most of which have been described by On & Holmes (1995), taking special note, where appropriate, of inoculum size as recommended in On & Holmes (1991). Acid production from carbohydrates was tested for in peptone yeast sugars. For motility determination, a blood-agar slope was inoculated with a broth suspension of the organism until a small amount of the inoculum remained at the base of the slope. Following overnight incubation, motility was determined from microscopic examination of a wet preparation of the broth at the base of the slope. Nitrate reduction was tested by adding Greiss–Ilosvay's reagents nos 1 and 2 (Merck) to a 24 h culture in nutrient broth no. 2 (Unipath) containing 1 % (w/v) KNO_2. Antibiotic-susceptibility tests were carried out by disc diffusion using cephalothin (30 μg) and nalidixic acid (30 μg) discs (Oxoid).

16S rRNA genes of the isolates were amplified by a PCR using universal primers pA (sequence 5′-AGAGTTTGATCCTGGCTCAG-3′, corresponding to positions 8–28 in *Escherichia coli* numbering) and pH (sequence 5′-AAGGAGGTGATCCAGCCGCA-3′, positions 1542–1522 in *E. coli* numbering) and directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the novel isolates were determined by searching the GenBank database using FASTA (Lipman & Pearson, 1985). These sequences and those of other known related strains were retrieved from GenBank and aligned with the newly determined sequences using the program DNATools (Rasmussen, 1995). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated with the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR, and the stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). For DNA–DNA hybridization, the bacterial cells were lysed and DNA was isolated and purified according to the method of Brenner *et al.* (1982). DNA from strain NCTC 12927^T was labelled with [^{32}P]dCTP by utilizing a nick translation kit (Invitrogen Life Technologies). This preparation was tested for reassociation to unlabelled DNA from the same strain and to the three other *Campylobacter* isolates as well as to DNAs from the type strains of *Campylobacter lari*, *C. jejuni* and *C. coli*. Relative binding ratios and divergence were calculated as described by Brenner *et al.* (1982). All reactions were done in duplicate at both the optimal temperature (50 °C) and the stringent temperature (65 °C). The clonality of the four *Campylobacter*-like organisms was investigated by amplified fragment length polymorphism (AFLP) fingerprinting for which DNA was extracted as previously described by Gibson *et al.* (1998). A single-enzyme AFLP (SAFLP) method was used in which the DNA was diluted to a concentration of 0·266 μg μl^{-1}. The SAFLP method was carried out as described by Champion *et al.* (2002) except that PCR amplification was performed in a Sprint thermal cycler (Hybaid). Cluster analysis was performed with BIONUMERICS (Applied Maths), using the Dice correlation coefficient and the UPGMA clustering algorithm.

Four strains of Gram-negative, comma-shaped bacilli morphologically similar to *Campylobacter* were isolated from rectal swabs taken from three common seals and the intestinal contents of a porpoise. The organisms were catalase-positive, oxidase-positive and motile. Colonies of all the isolates, when grown on CSBA at 37 °C in a microaerobic atmosphere, were 0·75–1·0 mm in diameter, circular, entire, low-convex, smooth, shiny, grey, translucent, butyrous and easily emulsified. Swarming did not occur. Growth did not occur at either 25 or 42 °C, nor did it occur in aerobic or anaerobic conditions. Growth did occur on unsupplemented nutrient agar and in the presence of 1 % (w/v) glycine but not in the presence of 2 or 3·5 % (w/v) NaCl. Urease was not produced. Nitrates were reduced but nitrites were not reduced. The strains were non-saccharolytic and were resistant to 30 μg cephalothin ml^{-1} and 30 μg nalidixic acid ml^{-1}. Hydrogen sulphide was produced in triple-sugar–iron (TSI) agar. Hippurate and indoxyl acetate were not hydrolysed.

To clarify the taxonomic interrelationships of the unidentified isolates, their 16S rRNA gene sequences were determined. The almost complete gene sequences of all four strains were elucidated and pairwise comparisons showed that all of the isolates were genetically highly related to each other, exhibiting 99·9–100 % sequence similarity (based on a comparison of approximately 1400 bases). The 16S rRNA gene sequence (>1400 nt) of a representative strain (NCTC 12927^T) was subjected to searches of GenBank/EMBL, which confirmed that the unknown bacterium was phylogenetically most closely related to the genus *Campylobacter* (data not shown). A tree constructed using the neighbour-joining method and depicting the phylogenetic position of the unidentified organism within the genus *Campylobacter* is shown in Fig. 1. The unknown bacterium formed a distinct subline within the genus, associated with a subcluster of species that included *C. jejuni* subsp. *jejuni* and subsp. *doylei*, *C. lari* and *C. coli*. Pairwise sequence comparisons revealed similarities of 98·8, 98·3 and 97·6 %, respectively, with the aforementioned species. Other *Campylobacter* species displayed substantially lower levels of similarity (data not shown).

In view of the high 16S rRNA gene sequence similarity between the unidentified isolates and *C. jejuni*, *C. lari* and *C. coli*, chromosomal DNA–DNA pairing was conducted. The results of these DNA–DNA hybridization studies are shown in Table 1. The four sea-mammal isolates displayed relative binding ratios >70 % under both optimal and

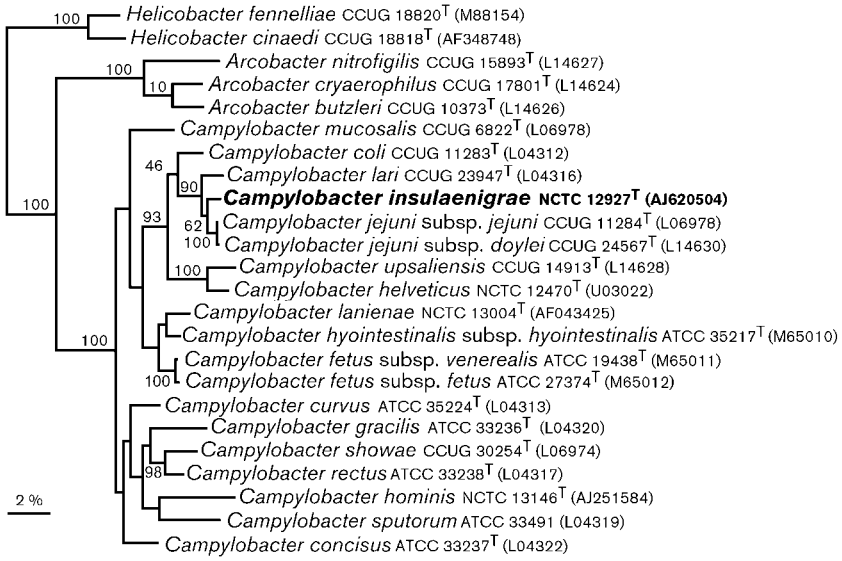

Fig. 1. Unrooted tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of *C. insulaenigrae* sp. nov. Bar, 2 % sequence divergence.

stringent hybridization conditions, thereby demonstrating that they are members of a single genomic species. This was consistent with very low divergence (%D) values. By contrast, *C. lari* displayed a relative binding ratio of 61 % under optimum conditions and 33 % under stringent conditions, with a D value of 11·7 % with respect to the reference organism (strain NCTC 12927^T). Significantly lower relative binding ratios and higher %D values were shown between the unknown bacterium and *C. jejuni* and *C. coli* (Table 1).

In the present study, we have comprehensively characterized four strains of a hitherto unknown bacterium recovered from three common seals and a porpoise in Scotland. It is evident from the results of the polyphasic taxonomic study that the unidentified *Campylobacter*-like isolates represent a novel *Campylobacter* species. Phylogenetically, the unknown bacterium shows close affinity with *C. jejuni*, *C. lari* and *C. coli*, displaying high 16S rRNA sequence similarity (approx. 1·2–2·4 % divergence). Despite the relatively high sequence similarities between the unidentified isolates and these species, DNA–DNA pairing studies show that the strains from sea mammals form a genetically homogeneous group and represent a distinct genomic species. The closest genetic relative to the unknown bacterium corresponds to *C. lari*, but the observed low relative binding ratio and high divergence (approx. 12 %) between strain NCTC 12927^T and the type strain of *C. lari* demonstrated that the bacterium from sea mammals represents a distinct species. It is known that *C. lari* is a phenotypically and genomically heterogeneous species. In a recent study by Duim *et al.* (2004), several groups were discerned within this species. The isolates recovered from marine mammals also resemble *C. lari* phenotypically. However, the novel species can be differentiated from *C. lari* by its inability to grow at 42 °C or in the presence of 2 % (w/v) NaCl. Therefore, on the basis of both phenotypic and molecular genetic evidence, we are of the opinion that the organisms from marine mammals merit classification as a novel species of the genus *Campylobacter*, for which the name *Campylobacter insulaenigrae* sp. nov. is proposed. Tests that serve to differentiate *C. insulaenigrae* from other members of the genus *Campylobacter* are shown in Table 2. It is pertinent to note that the four isolates of *C. insulaenigrae* were recovered from different animals and at different times. AFLP genetic profiling (Fig. 2) showed that the isolates were all genetically different from each other, thereby demonstrating that they represent different strains rather than a single clone.

Table 1. Relative binding ratio and divergence of DNA from *C. insulaenigrae* sp. nov. NCTC 12927^T

RBR, Relative binding ratio; %D, percentage divergence.

Source of unlabelled DNA	Results with labelled DNA from NCTC 12927^T		
	RBR at 50 °C	%D	RBR at 65 °C
C. insulaenigrae sp. nov. NCTC 12927^T	100	0·0	100
NCTC 12928	86	0·5	88
NCTC 12929	91	0·6	89
NCTC 12930	90	0·7	86
C. lari NCTC 11352^T	61	11·7	33
C. jejuni NCTC 11351^T	31	15·1	8
C. coli NCTC 11366^T	33	16·0	7

Description of *Campylobacter insulaenigrae* sp. nov.

Campylobacter insulaenigrae (in.su.lae.ni′grae. L. fem. n. *insula* isle/island; L. adj. *niger*, *-gra*, *-grum* black; N.L. gen. fem. n. *insulaenigrae* of the Black Isle, a region of northern Scotland).

Table 2. Differential characteristics of *C. insulaenigrae* sp. nov. and other members of the genus *Campylobacter*

Taxa: 1, *C. insulaenigrae* sp. nov.; 2, *C. coli*; 3, *C. concisus*; 4, *C. curvus*; 5, *C. fetus* subsp. *fetus*; 6, *C. fetus* subsp. *venerealis*; 7, *C. gracilis*; 8, *C. helveticus*; 9, *C. hyointestinalis* subsp. *hyointestinalis*; 10, *C. hyointestinalis* subsp. *lawsonii*; 11, *C. jejuni* subsp. *doylei*; 12, *C. jejuni* subsp. *jejuni*; 13, *C. lanienae*; 14, *C. lari*; 15, *C. mucosalis*; 16, *C. rectus*; 17, *C. showae*; 18, *C. sputorum*; 19, *C. upsaliensis*. Data are from this study, On *et al.* (1996) and Logan *et al.* (2000). Symbols: +, 90–100 % of strains positive; (+), 80–89 % of strains positive; d, 21–79 % of strains positive; (−), 11–20 % of strains positive; −, 0–10 % of strains positive; W, weak.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Catalase	+	+	−	−	+	(+)	d	−	+	+	d	+	+	+	−	(−)	+	d	−
Growth at/in:																			
25 °C	−	−	−	−	+	+	−	−	(−)	−	−	−	−	−	−	−	−	−	−
42 °C	−	+	d	d	(+)	−	d	+	+	+	−	+	+	+	+	(−)	d	+	(+)
1 % Glycine	+	+	d	+	+	−	+	d	+	d	d	+	−	+	d	+	d	+	+
2 % NaCl	−	−	d	d	−	−	d	(−)	−	−	−	−	−	(+)	+	d	+	+	−
Oxidase	+	+	d	+	+	+	−	+	+	+	+	+	+	+	+	+	d	+	+
Nitrate reduction	+	+	(−)	+	+	+	(+)	+	+	+	−	+	+	+	−	+	+	+	+
H_2S in TSI	−	d	−	(−)	−	−	−	−	+	+	−	−	−	−	+	−	d	+	−
Indoxyl acetate hydrolysis	−	+	−	d	−	−	d	+	−	−	+	+	−	−	−	+	d	−	+
Hippurate hydrolysis	−	−	−	(−)	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
Microaerobic growth	+	+	+	d	+	+	−	+	+	+	+	+	+	+	+	−	d	+	+
Anaerobic growth	−	−	+	+	d	d	+	−	−	+	−	−	W	−	+	+	+	+	−

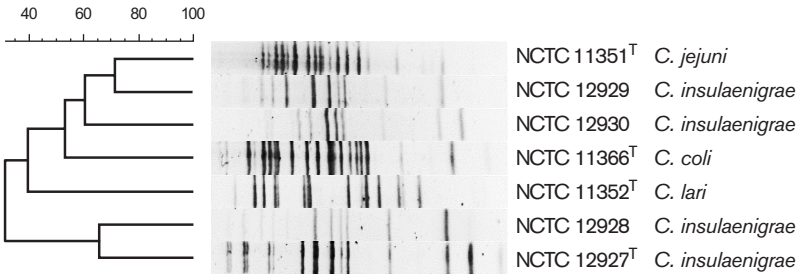

Fig. 2. Dendrogram of SAFLP profiles digested with *Hin*dIII. Cluster analysis was performed with BIONUMERICS (Applied Maths), using the Dice correlation coefficient and the UPGMA clustering algorithm.

The following description of morphological and physiological characteristics is based on the results of studies of four strains. Gram-negative, motile, non-encapsulated, non-spore-forming, comma-shaped rods. Colonies on CSBA incubated in a microaerobic atmosphere at 37 °C for 48 h are 0·75–1·0 mm in diameter, circular, entire, low-convex, smooth, shiny, grey, translucent, butyrous and easily emulsified. Growth does not occur at 25 or 42 °C, or in aerobic or anaerobic atmospheres. Growth occurs in the presence of 1 % (w/v) glycine but not in the presence of 2 or 3·5 % (w/v) NaCl. Oxidase- and catalase-positive, but urease-negative. Nitrates are reduced, but nitrites are not reduced. H_2S is produced in TSI agar, but hippurate and indoxyl acetate are not hydrolysed. Resistant to 30 μg cephalothin and 30 μg nalidixic acid ml^{-1}.

The type strain is NCTC 12927^T (=CCUG 48653^T). Isolated from marine mammals.

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