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Campylobacter lanienae sp. nov., a new species isolated from workers in an abattoir

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***Campylobacter*-like organisms were isolated from the faeces of healthy individuals during a hygiene survey of abattoir workers. The strains, which exhibited characteristics of *Campylobacter*, being non-glucose-fermenting, oxidase- and catalase-positive, Gram-negative, motile rods, were identified to the genus level by a PCR assay. Nucleotide sequence analysis of the 16S rRNA gene, DNA homology experiments and determination of G+C content demonstrated that they constituted a previously undescribed species, whose nearest phylogenetic neighbours were *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter fetus* and *Campylobacter mucosalis*. The name *Campylobacter lanienae* sp. nov. is proposed for this taxon and species-specific PCR primers were evaluated which will find use in the study of its epidemiology, prevalence and pathogenicity.**

Keywords: *Campylobacter*, phylogenetic study, human enteric isolates

INTRODUCTION

The ε-subclass of the *Proteobacteria* contains the genera *Campylobacter*, *Helicobacter*, *Arcobacter* and *Wolinella* (Vandamme *et al*., 1991). *Campylobacter jejuni* and *Campylobacter coli* are together the most common causative agents of bacterial enteritis in man (Tauxe, 1992). The role of certain other *Campylobacter* species in human disease remains to be definitively established, and it is possible that their importance may be currently underestimated.

The species of *Campylobacter* occupy diverse ecological niches. *C. jejuni*, *C. coli*, *Campylobacter lari* and *Campylobacter upsaliensis* are recognized agents of diarrhoeal disease in man, although their primary hosts are animals. There is evidence that *Campylobacter hyointestinalis*, a causative agent of proliferative porcine enteritis (Gebhart *et al*., 1985) can also be an agent of human gastroenteritis (Linton *et al*., 1997; Lawson *et al*., 1998). *Campylobacter fetus* is occasionally found as an agent of bacteraemia of the elderly or immunocompromised. *Campylobacter sputorum* bv. sputorum and *C. sputorum* bv. paraureolyticus have also been isolated from cases of human diarrhoea (On *et al*., 1998). Several *Campylobacter* species can be isolated from the human mouth – they include *Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter gracilis*, *Campylobacter rectus* and *Campylobacter showae*, which are associated with the gingival crevice and may be implicated in periodontal disease. *Campylobacter mucosalis*, *Campylobacter helveticus* and *C. sputorum* bv. fecalis are all found in animal hosts and are not considered to be significant human pathogens (Skirrow, 1994).

During a routine hygiene screen of asymptomatic abattoir workers, campylobacter-like organisms (CLOs) were cultured from stool specimens of two individuals. In the present report we provide molecular and phenotypic evidence that these CLOs belong to a previously undescribed species of the genus *Campylobacter*.

METHODS

Initial isolation. Faecal samples provided by abattoir workers for a routine hygiene survey were screened for bacterial enteric pathogens, including *Campylobacter* species. The faeces were inoculated onto Campylosel agar (bioMérieux), consisting of 5% (v/v) blood in Columbia agar base with the selective antibiotics cefoperazone (32 mg

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Abbreviation: CLO, campylobacter-like organism.

The GenBank accession numbers for the sequences reported in this paper are: AF043425 (NCTC 13004^T), AF043423 (UB 993), AF043422 (UB 992) and AF043424 (UB 994).

Table 1. Bacterial strains and 16S rRNA sequences

Bacteria	Source	Strain no.*	GenBank no.
Campylobacter coli	Porcine	NCTC 11366^T	L04312
Campylobacter concisus	Human	NCTC 11485^T	L04322
Campylobacter curvus	Human	NCTC 11649^T	L04313
Campylobacter fetus subsp. *fetus*	Ovine	NCTC 10842^T	M65012
Campylobacter fetus subsp. *venerealis*	Bovine	NCTC 10354^T	M65011
Campylobacter gracilis	Human	NCTC 12738^T	L04320
Campylobacter helveticus	Feline (cat)	NCTC 12470^T	U03022
Campylobacter hyoilei (*C. coli*)	Porcine	RMIT 32A^T	L19738
Campylobacter hyointestinalis subsp. *hyointestinalis*	Porcine	NCTC 11608^T	M65010
Campylobacter jejuni subsp. *jejuni*	Bovine	NCTC 11351^T	L04315
Campylobacter jejuni subsp. *doylei*	Human	NCTC 11951^T	L14630
Campylobacter lari	Avian (gull)	NCTC 11352^T	L04316
Campylobacter mucosalis	Porcine	NCTC 11000^T	L06978
Campylobacter rectus	Human	NCTC 11489^T	L04317
Campylobacter showae	Human	NCTC 12843^T	L06974
Campylobacter sputorum bv. bubulus	Bovine	NCTC 11367^T	L04319
Campylobacter sputorum bv. fecalis	Ovine	NCTC 11415^T	–
Campylobacter sputorum bv. sputorum	Human	NCTC 11528^T	–
Campylobacter upsaliensis	Canine	NCTC 11541^T	L14628
Bacteroides ureolyticus	Human	NCTC 10941^T	L04321
AW strain	Human	NCTC 13004^T	AF043425†
AW strain	Human	UB 993	AF043423†
Arcobacter butzleri	Human	NCTC 12481^T	L14626
Arcobacter cryaerophilus	Bovine	NCTC 11885^T	L14624
Arcobacter nitrofigilis	Plant	NCTC 12251^T	L14627
Arcobacter skirrowii	Ovine	NCTC 12713^T	L16625
Helicobacter acinonychis	Feline (cheetah)	NCTC 12686^T	M88148
Helicobacter bilis	Murine (mouse)	ATCC 51630^T	U18766
Helicobacter canis	Canine	NCTC 12739^T	L13464
Helicobacter cinaedi	Human	NCTC 12423^T	M88150
Helicobacter fennelliae	Human	NCTC 11612^T	M88154
Helicobacter felis	Feline (cat)	NCTC 12436^T	M37642
Helicobacter hepaticus	Murine (mouse)	ATCC 51448^T	U07574
Helicobacter muridarum	Murine (rat)	NCTC 12714^T	M80205
Helicobacter mustelae	Musteline (ferret)	NCTC 12198^T	M35048
Helicobacter nemestrinae	Primate (macaque)	NCTC 12491^T	X67854
Helicobacter pametensis	Avian (tern)	ATCC 51478^T	M88147
Helicobacter pylori	Human	NCTC 11637^T	M88157
Helicobacter pullorum	Avian (chicken)	NCTC 12824^T	L36141
CLO-3‡	Human	NCTC 12462	M88151
'*Flexispira rappini*'‡	Human	NCTC 12461^T	M88137
'*Gastrospirillum hominis*'§	Human	Uncultivable	L10079
Wolinella succinogenes	Bovine	NCTC 11488^T	M88159
Escherichia coli		NCTC 9001^T	J01695

* NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; ATCC, American Type Culture Collection, Manassas, VA, USA; RMIT, Royal Melbourne Institiute of Technology, Melbourne, Australia.

† Sequenced in this study.

‡ Species identified as *Helicobacter* by 16S rRNA analysis (Stanley *et al*., 1993).

§ Species identified as *Helicobacter* by 16S rRNA analysis (Solnick *et al*., 1993).

Table 2. Phenotypic characteristics differentiating *C. lanienae* from other *Campylobacter* species

Data were obtained from Vandamme & De Ley (1991) with the following exceptions: *Campylobacter gracilis* (Tanner *et al.*, 1981), *Campylobacter showae* (Etoh *et al.*, 1993), *Campylobacter helveticus* (Stanley *et al.*, 1992) and *Campylobacter hyoilei* (*C. coli*) (Alderton *et al.*, 1995). Test results: +, positive reaction; −, negative reaction; w, weak reaction; v, variable reaction; R, resistant; S, sensitive; ND, not determined.

Species	Catalase	Nitrate reduction	Nitrite reduction	H_2S production (TSI)	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Growth at:		Growth in 1% glycine	Alkaline phosphatase*	Susceptibility to:†		G+C content (mol %)
							25 °C	42 °C			NA	C	
Campylobacter lanienae	+	+	+	−	−	−	−	+	−	+	R	R	36
Campylobacter hyointestinalis subsp. *hyointestinalis*	+	+	−	+	−	−	v	+	+	v	R	S	33–36
Campylobacter fetus subsp. *venerealis*	+	+	−	−	−	−	+	−	−	−	R	S	33–34
Campylobacter fetus subsp. *fetus*	+	+	−	−	−	−	+	−	+	−	R	S	33–35
Campylobacter mucosalis	−	+	+	+	−	−	−	+	+	v	R	S	36–38
Campylobacter concisus	−	+	+	+	−	−	−	+	+	v	R	R	37–41
Campylobacter curvus	−	+	+	+	−	+	−	+	+	ND	S	ND	45–46
Campylobacter sputorum bv. bubulus	−	+	+	+	−	−	−	+	+	−	R	S	29–30
Campylobacter sputorum bv. fecalis	+	+	+	+	−	−	−	+	+	v	R	S	30–32
Campylobacter sputorum bv. sputorum	−	+	+	+	−	−	−	+	+	ND	S	S	30–31
Campylobacter gracilis	−	+	+	ND	ND	ND	ND	ND	ND	ND	R	ND	44–46
Campylobacter rectus	−	+	+	+	−	+	−	w	+	ND	S	ND	45–46
Campylobacter showae	+	+	+	+	−	+	−	+	v	−	R	S	44–46
Campylobacter upsaliensis	w/−	+	−	−	−	+	−	+	v	v	S	S	32–36
Campylobacter helveticus	−	+	ND	−	−	+	−	+	+	−	S	S	34
Campylobacter coli	+	+	−	−	−	+	−	+	+	v	S	R	30–33
Campylobacter lari	+	+	−	−	−	−	−	+	+	−	R	R	30–32
Campylobacter hyoilei (*C. coli*)	+	+	+	+	−	ND	ND	v	+	ND	S	R	35
Campylobacter jejuni subsp. *doylei*	+	−	−	−	v	+	−	−	+	+	S	S	30–31
Campylobacter jejuni subsp. *jejuni*	+	+	−	−	+	+	−	+	+	+	S	R	30–33

* Obtained from bioMérieux.

† NA, nalidixic acid; C, cephalothin.

l^{-1}), vancomycin (10 mg l^{-1}) and amphotericin B (3 mg l^{-1}). These plates were incubated for 48 h at 37 °C in a microaerobic atmosphere of 5% O_2, 5% CO_2, 2% H_2, 88% N_2 (by vol.).

Bacterial strains and culture conditions. The reference strains of *Campylobacter*, *Arcobacter*, *Helicobacter*, *Wolinella* and *Escherichia coli* used for sequence analysis are listed in Table 1. Two new CLOs, the subject of this study, were provisionally termed the 'AW strains', (abattoir worker strains). They were cultured on 5% (v/v) Columbia blood agar as previously described (Stanley *et al.*, 1993).

Phenotypic characterization. The phenotype of the isolates was determined using recommended media and methodologies (Burnens & Nicolet, 1993; On & Holmes, 1991, 1992). Additional tests were performed as follows. Production of extracellular deoxyribonuclease (DNase) was determined by the method of Lior & Patel (1987). Colony morphology was recorded after 3 d microaerobic incubation on 5% (v/v) blood in Columbia agar base at 37 °C. All tests were performed in triplicate, on separate occasions and with freshly prepared media. Tests useful for the differentiation of *C. lanienae* and other campylobacters are summarized in Table 2.

Electron microscopy. Cells were taken from a 48 h culture on blood agar and resuspended in a 1% (v/v) formalin solution. A Formvar-coated grid was placed on a drop of the bacteria/formalin suspension for 2 min, then transferred to a drop of 2% (w/v) ammonium molybdate solution for a further 2 min. Grids were examined at a magnification of ×13 500 in a Phillips EM420 electron microscope at 80 kV.

Nucleic acid techniques. Preparation of genomic DNA was as previously described (Stanley *et al.*, 1992). The G+C content was determined by thermal denaturation (Owen & Pitcher, 1985). DNA–DNA slot-blot hybridization was performed for the AW strains, the *Campylobacter* species listed in Table 2, *Arcobacter butzleri* and *Helicobacter pylori*, employing NCTC 13004^T as a probe. The method was as described previously (Stanley *et al.*, 1992), except that a digoxigenin (DIG) High Prime labelling and detection kit was used (Roche), hybridization was performed under both optimal [2× SSC (0·3 M sodium chloride, 0·03 M sodium citrate) at 64 °C] and stringent (0·1× SSC at 64 °C) renaturation conditions and density analysis was performed using an Agfa scanner and Scan Analysis software (version 2.21; Biosoft) to determine relative homology values.

A 1500 bp fragment of the 16S rRNA gene of the AW strains (NCTC 13004^T and UB 993) and two further isolates were amplified by PCR as previously described (Stanley *et al.*, 1993). Amplicons were purified and sequenced according to the manufacturer's instructions for the ABI Prism sequencing kit (Perkin Elmer).

Phylogenetic analysis of 16S rRNA gene sequence. The 16S rRNA gene sequences for NCTC 13004^T and UB 993 were aligned with the data for 17 *Campylobacter*, 4 *Arcobacter*, 16

Helicobacter, *Bacteroides ureolyticus*, *Wolinella succinogenes* and *E. coli* sequences. A dissimilarity matrix was constructed from aligned sequences and data were corrected for multiple base changes by the method of Jukes & Cantor (1969). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the TREECON package (Van de Peer & De Wachter, 1993). Bootstrap analysis (1000 replicate samples) was carried out within TREECON.

PCR identification to genus level. PCR primers for the identification of the genus *Campylobacter* were as previously described (Linton *et al.*, 1996). Primers for the identification of the genera *Arcobacter* and *Helicobacter* were designed from alignment of sequences retrieved from GenBank (see above) with the Multalign program (Corpet, 1988)

RESULTS

Isolation and culture characteristics of strains

Strains NCTC 13004^T and UB 993 were isolated from separate workers. On initial isolation and subsequent culture, colonies of these strains were *Campylobacter*-like, but growth was poor in comparison to *C. jejuni*. Microscopic examination revealed slender, curved, Gram-negative rods which were motile in wet mounts. They gave an oxidase-positive reaction, failed to metabolize or produce acid from glucose, were sensitive to polymyxin B and were negative for arylsulfatase and pyrazinamidase. Together, the above features are characteristic of the genus *Campylobacter* (Burnens & Nicolet, 1993). They showed further general *Campylobacter* characteristics of catalase positivity, resistance to nalidixic acid and growth at 42 °C.

NCTC 13004^T and UB 993 could be distinguished from other *Campylobacter* species by several standard phenotypic tests, as outlined in Table 2 (see also species description). They were resistant to cephalothin and did not produce DNase.

Electron microscope observation of NCTC 13004^T

Cells were slender with a slight curvature. Flagella were unsheathed. They were single and bipolar (Fig. 1).

DNA base composition and DNA–DNA hybridization

The DNA base composition of NCTC 13004^T and UB 993 was determined as 36 mol% G+C. DNA–DNA hybridization experiments confirmed 100% homology between the two strains. There was no detectable

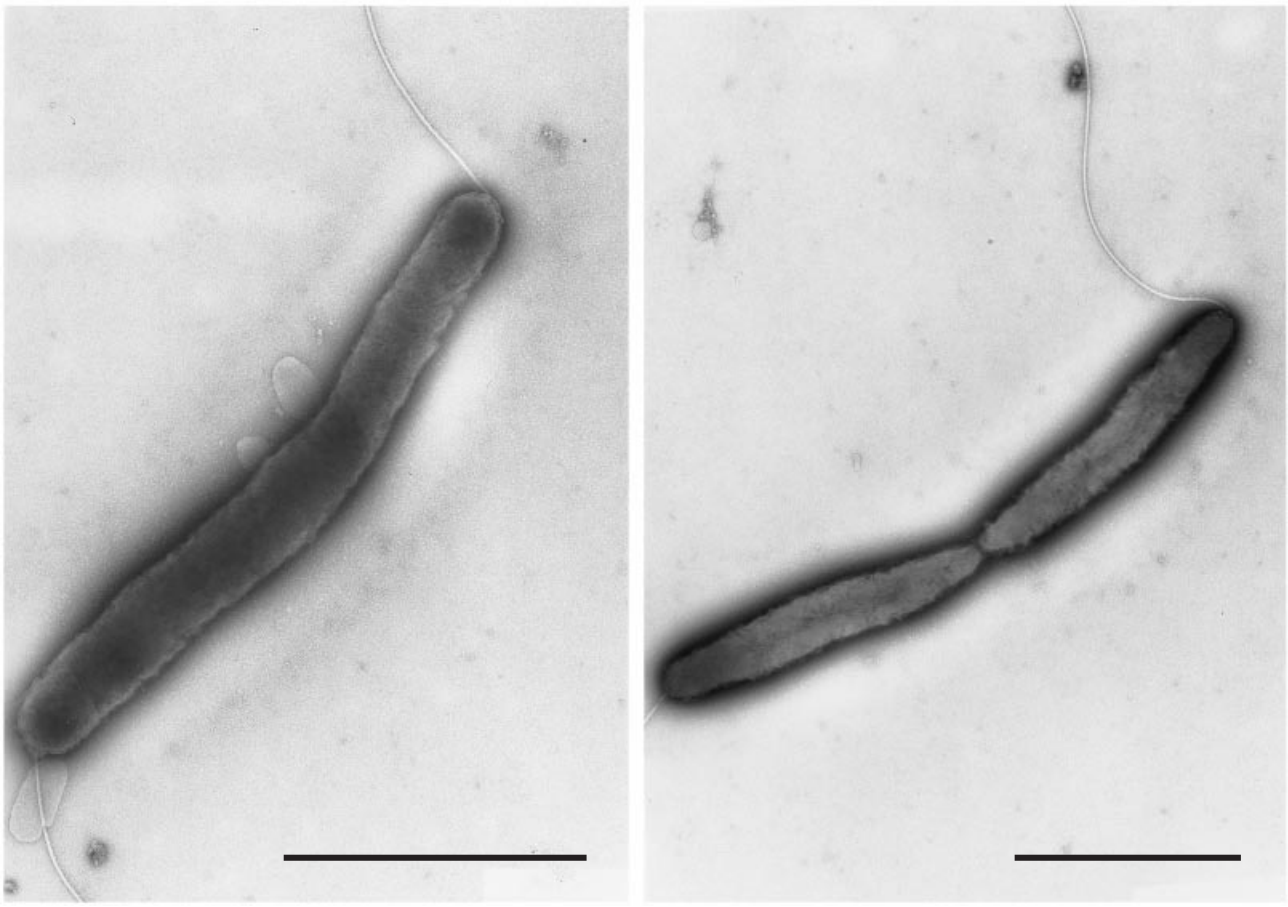

Fig. 1. Electron micrographs of *C. lanienae* NCTC 13004^T. Cells vary in length from 1·2 to 2·4 μm. The organism exhibits a slight spiral curvature and carries single bipolar unsheathed flagella. Bar, 1 μm.

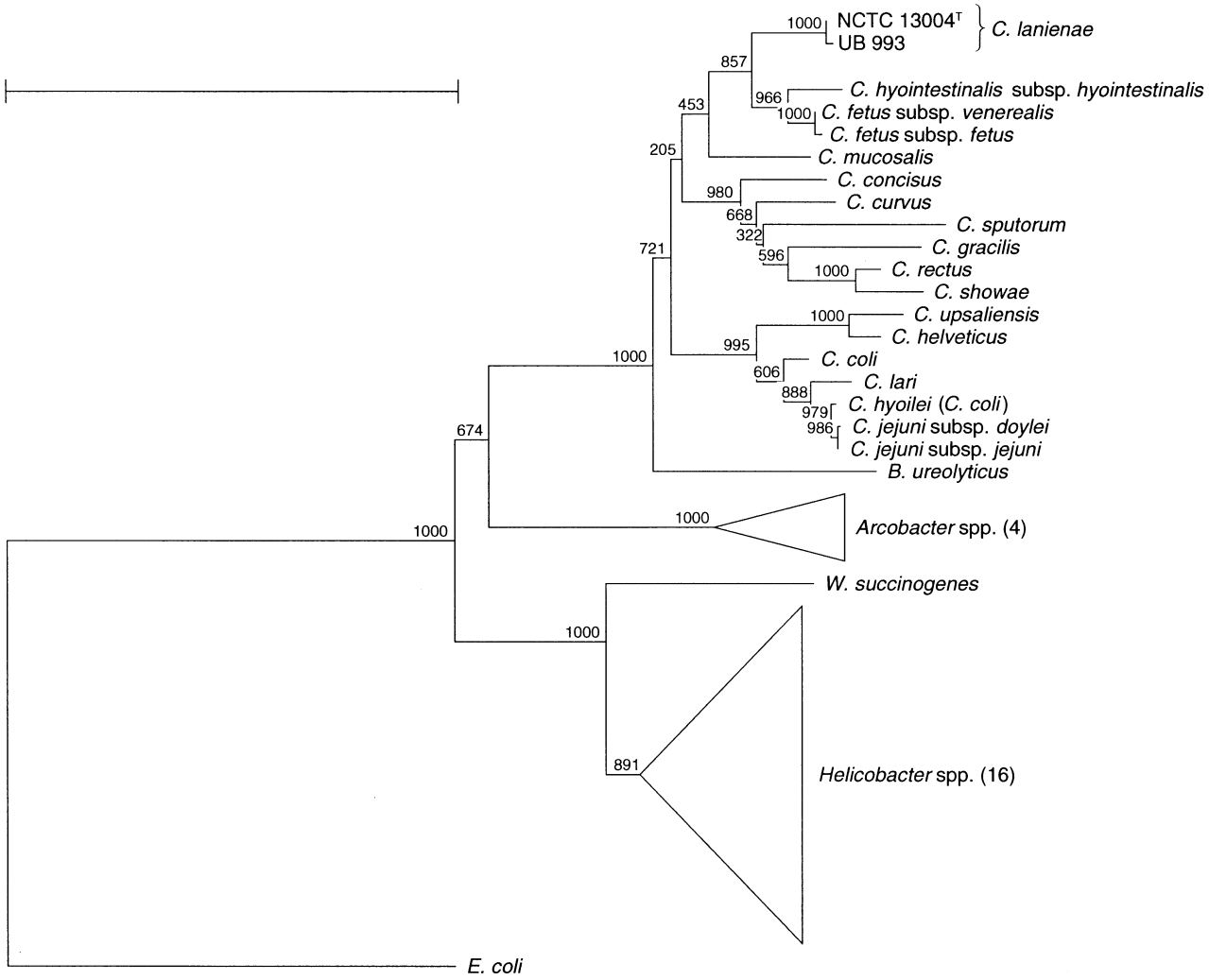

Fig. 2. Phylogenetic position of *C. lanienae* sp. nov. Neighbour-joining tree based on analysis of 16S rRNA gene sequences. Bar represents 0·1 nucleotide substitutions per base. Bootstrap supporting values are shown for each branch point. Numbers in parentheses are the number of species analysed.

homology with *Arcobacter* (*butzleri*), *Helicobacter* (*pylori*) or any *Campylobacter* species (see Table 2) except *C. hyointestinalis* subsp. *hyointestinalis*. *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis* and *C. mucosalis* (all less than 20% homology, even at the optimum renaturation temperature).

Assignment of strains to genus level by PCR

On the basis of the known 16S rRNA gene sequences for species in the ε-subclass of the *Proteobacteria*, PCR primers were designed for rapid identification of the genera *Arcobacter* and *Helicobacter*. The forward and reverse primers for identification of *Arcobacter* were: A393F (5′-ACA ATG GAC GAA AGT CTG AT-3′), located between nucleotides 393 and 413, and A1151R (5′-CAC CTT CCT CCT ACT TGC GT-3′), located between nucleotides 1151 and 1171. The forward and reverse primers for the identification of *Helicobacter* were: H297F (5′-GGC TAT GAC GGG TAT CCG GC-3′), located between nucleotides 297 and 307, and H1026R (5′-GCC GTG CAG CAC CTG TTT TC-3′) located between nucleotides 1026 and 1046. Genomic DNA extracted from AW strains by standard procedures (Wilson, 1987) was tested with the above primers and a PCR specific for the genus *Campylobacter* (Linton *et al.*, 1996). They did not produce an amplicon with *Arcobacter*- and *Helicobacter*-specific primers, but produced an amplicon of predicted size with primers specific for *Campylobacter*.

Sequence of the 16S rRNA gene and phylogenetic analysis

The sequence of the 16S rRNA (small subunit) gene was determined for NCTC 13004^T and UB 993 [GenBank accession numbers: AF043425 (NCTC 13004^T) and AF043423 (UB 993)]. When these sequences were aligned with 16S rRNA gene sequences representative of the genera *Campylobacter*, *Arco-*

bacter and *Helicobacter*, the strains clearly fell within *Campylobacter*.

A sequence dissimilarity matrix was constructed for these sequences; between them and those of *Campylobacter*, *Arcobacter* and *Helicobacter* spp.; and between them and that of *E. coli* (data not shown). The percentage dissimilarity between NCTC 13004^T and UB 993 was 0·2%. Dissimilarities between NCTC 13004^T and the most closely related known species were as follows: *C. hyointestinalis* subsp. *hyointestinalis*, 3·42%; *C. fetus* subsp. *venerealis*, 3·89%; *C. fetus* subsp. *fetus*, 3·99%; and *C. mucosalis*, 5·05%. The percentage dissimilarity between NCTC 13004^T and *C. jejuni* was 5·62%; between NCTC 13004^T and *Arcobacter* spp., it was at least 14·55%; and between NCTC 13004^T and various *Helicobacter* spp., it varied from 14·17 to 16·82%.

Distance data were corrected for multiple base changes by the method of Jukes & Cantor (1969) and used to generate a neighbour-joining phylogenetic tree, shown in Fig. 2. This may be usefully compared with Table 2 (phenotypic characteristics), which is ordered according to established phylogenetic relationships. NCTC 13004^T and UB 993 represent a distinct monophyletic lineage within *Campylobacter*; this branching was supported by a bootstrap value of 1000. We termed the new taxon *Campylobacter lanienae* sp. nov.

PCR primers specific for *C. lanienae* sp. nov.

Primers were designed based on alignments of the 16S rRNA gene sequences of the two *C. lanienae* strains with all known *Campylobacter*, *Helicobacter* and *Arcobacter* spp. The forward primer CLAN76F (5′-GTA AGA GCT TGC TCT TAT GAG-3′) was located between nucleotides 76 and 96, and the reverse primer CLAN1021R (5′- TCT TAT CTC TAA GAG GTT CTT A-3′) was located between nucleotides 1021 and 1001. The predicted PCR amplicon size was 920 bp and the optimum annealing temperature was 58 °C. Amplicons were produced from NCTC 13004^T and UB 993, but not from type strains of any other *Campylobacter* species.

Status of other CLO isolates from abattoir workers

Two further oxidase- and catalase-positive CLOs (UB 992, UB 994) had been isolated from other workers screened in the same hygiene survey. These later tested positive with the species-specfic PCR primers above, and amplicons from them were sequenced ['*C. lanienae*-like'; GenBank accession numbers: AF043422 (UB 992), AF043424 (UB 994)]. They differed from NCTC 13004^T by 9 out of 1463 nucleotides (0·6%). These isolates were not further characterized.

DISCUSSION

In the present report we have fully characterized two *Campylobacter* strains isolated from abattoir workers in Switzerland, showing that they belong to a previously undescribed species, for which we propose the name *Campylobacter lanienae* sp. nov. Its bacteriological characteristics and cell morphology, including unsheathed polar flagella, are typical for *Campylobacter*. DNA–DNA hybridization studies indicated that there was no significant homology with any other species of *Campylobacter*, *Helicobacter* or *Arcobacter*. Sequence analyses of the 16S rRNA gene were consistent with a new species (bootstrap supporting value 1000) whose nearest phylogenetic neighbours are *C. hyointestinalis* subsp. *hyointestinalis*, *C. fetus* and *C. mucosalis*. The hosts of these species are farm animals. The phylogenetic relationship is borne out by the G+C content of *C. lanienae*, which falls within or close to the range of *C. fetus*, *C. hyointestinalis* subsp. *hyointestinalis* and *C. mucosalis* (cf. Fig. 2 and Table 2). In terms of biochemical differentiation, *C. lanienae* can be distinguished from both subspecies of *C. fetus* by its failure to grow at 25 °C, ability to grow at 42 °C and alkaline phosphatase activity. It can be distinguished from *C. hyointestinalis* subsp. *hyointestinalis* or *C. mucosalis* by its failure to produce H_2S from TSI agar stabs or to grow in 1% glycine. These three known species are not commonly implicated in human illness and we note that the human carriers of *C. lanienae* showed no sign of gastrointestinal illness.

NCTC 13004^T was isolated from a 21-year-old healthy abattoir worker screened for enteric pathogens prior to working in another abattoir. He had been previously exposed to cattle and pig carcasses. The other worker (age not recorded) had been exposed mainly to cattle carcasses. Their only common exposure factor was work at an abattoir. Although it might be reasonable to suppose that either cattle or pigs are the hosts of this new species, further investigations by the Swiss National Reference Laboratory for Foodborne Diseases have as yet failed to produce an isolate of this species from an animal source (cattle, pigs, dogs, cats, sheep, poultry, mice). Further analysis of human carriage would be justified, for example through studying its occurrence in the faeces of patients with acute gastroenteritis, workers in animal husbandry and the meat industry, and healthy controls. Molecular ecological studies should be facilitated by application of the species-specific PCR primers described herein, as demonstrated by preliminary speciation of two further isolates from the same hygiene survey.

Description of *Campylobacter lanienae* sp. nov.

Campylobacter lanienae (lan.i.en′ae. L. n. *laniena* abattoir, after place of work of human carriers from whom first isolated).

Gram-negative, non-spore-forming rods, 1·2–2·4 μm in length at 48 h. Cells are regular and slender, slightly spiral and with rounded ends. Darting motility in hanging-drop preparations. Single bipolar flagella are unsheathed. After 3 d microaerobic incubation at 37 °C, colonies on agar are 0·5 mm in diameter,

smooth, entire, translucent and cause some greening of blood agar. Microaerophilic, but grows weakly under anaerobic conditions. Grows at 37 and 42, but not at 25 °C. Glucose not fermented. Urease, DNase, arylsulfatase and pyrazinamidase not produced. Oxidase, catalase and alkaline phosphatase produced. Nitrate and nitrite reduced. Hydrogen sulfide not produced from triple-sugar iron medium. Indoxyl acetate and hippurate not hydrolysed. No growth in 1 % glycine or 1·5 % NaCl. Resistant to nalidixic acid and cephalothin; sensitive to polymyxin B. G+C content of genomic DNA by thermal denaturation is 36 mol %. Isolated from faecal samples from healthy humans working in an abattoir. Pathogenicity unknown. Type strain is NCTC 13004^T.

Formal description of the type strain. NCTC 13004^T is the type strain of *C. lanienae* and conforms to the species description given above. It was isolated in Switzerland from an asymptomatic individual working in an abattoir.

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