Emended description of *Campylobacter sputorum* and revision of its infrasubspecific (biovar) divisions, including *C. sputorum* biovar *paraureolyticus*, a urease-producing variant from cattle and humans

S. L. W. On,¹ H. I. Atabay,² J. E. L. Corry,² C. S. Harrington^{1,3}
and P. Vandamme^{4,5}

Author for correspondence: S. L. W. On. Tel: +45 35 30 01 00. Fax: +45 35 30 01 20.
e-mail: sto@svs.dk

¹ Danish Veterinary Laboratory, Bülowsvej 27, DK-1790 Copenhagen V, Denmark

² Department of Veterinary Science, University of Bristol, Bristol, UK

³ Department of Medical Microbiology, University of Aberdeen, Aberdeen, UK

⁴ Laboratorium voor Microbiologie, University of Ghent, B-9000, Ghent, Belgium

⁵ Department of Medical Microbiology, University of Antwerp, B-2610 Antwerp, Belgium

A polyphasic taxonomic study of 15 bovine and human strains assigned to the catalase-negative, urease-positive campylobacter (CNUPC) group identified these bacteria as a novel, ureolytic biovar of *Campylobacter sputorum* for which we propose the name *C. sputorum* bv. *paraureolyticus*: suitable reference strains are LMG 11764 (human isolate) and LMG 17590 (= CCUG 37579, bovine isolate). The present study confirmed previous findings showing that the salient biochemical tests used to differentiate *C. sputorum* bv. *sputorum* from *C. sputorum* bv. *bubulus* are not reproducible; and that the absolute validity of source-specific biovars of the species is questionable. A correlation between the results of numerical analysis of protein profiles and the reaction of strains in certain enzyme tests was, however, noted. Therefore, it is proposed that the infrasubspecific (biovar) divisions of *C. sputorum* should be revised to include bv. *sputorum* for catalase-negative strains; bv. *fecalis* for catalase-positive strains; and bv. *paraureolyticus* for urease-positive strains. Strains classified previously as bv. *bubulus* should be reclassified as bv. *sputorum*. The species description of *C. sputorum* is revised accordingly.

Keywords: *Campylobacter sputorum* biovar *paraureolyticus*

INTRODUCTION

The genus *Campylobacter* was first proposed in 1963 and included two species, *Campylobacter fetus* and *Campylobacter bubulus* (36). This classification has since been extensively revised due to research demonstrating the ecological diversity and clinical importance of *Campylobacter* spp. [reviewed by (37)]; and also as a result of the use of improved taxonomic methods to elucidate interstrain relationships (11, 21, 41–46). Consequently, [*Vibrio*] *sputorum* (32) and [*Vibrio fecalis*] (9) were assigned to the genus *Campylobacter* on the basis of DNA base composition and

phenotypic characters (5, 46). Although *Campylobacter sputorum* and *C. bubulus* were initially considered related at the subspecific level (46), subsequent DNA homology studies clearly showed these taxa, and ‘*Campylobacter fecalis*’ also, to belong to the same species, *C. sputorum* (13, 33, 39). Observations correlating biochemical differences between strains isolated from humans, the bovine and ovine genital tract and ovine faeces led to the infrasubspecific division of *C. sputorum* into three biovars, bv. *sputorum*, bv. *bubulus* and bv. *fecalis* (33). Conversely, an organism first isolated from pigs affected by proliferative enteritis and named *C. sputorum* subsp. *mucosalis* (17) demonstrated negligible levels of DNA–DNA homology to *C. sputorum*, and was thus considered a separate species, *Campylobacter mucosalis* (34). The status of *C. sputorum* and *C. mucosalis* as distinct species has since been extensively validated by various phenotypic and genotypic analyses (7, 22, 26, 40–41, 43).

Abbreviations: CNUPC, catalase-negative, urease-positive campylobacter; H₂S/TSI, hydrogen sulfide production in triple sugar iron; S-level, similarity level; UPGMA, unweighted pair group method with arithmetic averages.

The GenBank accession number for the BU 112B 16S rDNA sequence reported in this paper is AF022768.

Table 1. Strains used

Square brackets indicate that the species is generically misnamed and is regarded as a species *incertae sedis* (41). Previous designations are given in parentheses in the third column. Abbreviations: BU, Bristol University, Bristol, UK; CCUG, Culture Collection of the University of Göteborg, Sweden; CHY, *C. hyointestinalis*-like strain; CNUPC, catalase-negative, urease-positive campylobacter; LMG, Culture Collection, Laboratorium voor Microbiologie, Ghent, Belgium; NCTC, National Collection of Type Cultures, London, UK; ^T, type strain.

Organism	Strain number	Additional information	Clinical and geographical origin (if known)
<i>Campylobacter</i> species			
<i>C. sputorum</i>	LMG 11764	bv. paraureolyticus	Human diarrhoea, Canada
	LMG 17590 (= BU 112B, CCUG 37579)	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	LMG 17593 (= BU 308B)	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	LMG 17589 (= BU 464B)	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	LMG 17592 (= BU 31C)	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	LMG 17591 (= BU 73C)	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 150B	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 168B	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 751B	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 90B	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 132B	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 179B	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 333C	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 651C	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	BU 86C (= CCUG 37580)	bv. paraureolyticus (CNUPC group)	Bovine faeces, Bristol, UK
	LMG 7795 ^T (= NCTC 11528 ^T)	bv. sputorum	Human oral cavity, USA
	LMG 11761	bv. sputorum	Human, Canada
	LMG 11765	bv. sputorum (bv. bubulus)	Human, Canada
	LMG 14261	bv. sputorum	Human, pus from pressure sore, Belgium
	LMG 7975 (= CCUG 20706)	bv. sputorum	Porcine intestine, Preston, UK
	LMG 10388 (= CCUG 24261)	bv. sputorum	Ovine foetus, stillborn, Huddinge, Sweden
	LMG 8535 (= CCUG 22572)	bv. sputorum	Ovine faeces, Montana, USA
	LMG 6447 (= NCTC 11367)	bv. sputorum (bv. bubulus)	Bovine semen, Brussels, Belgium
	LMG 8531 (= NCTC 11415)	bv. fecalis	Ovine faeces, Toronto, Canada
	LMG 8532 (= CCUG 20703)	bv. fecalis	Bovine faeces, Preston, UK
	LMG 8534 (= CCUG 22571)	bv. fecalis	Ovine faeces, Montana, USA

Table 1 (cont.)

Organism	Strain number	Additional information	Clinical and geographical origin (if known)
<i>C. coli</i>	LMG 6728 (= CCUG 17695B)	bv. fecalis	Ovine faeces, geographic source unknown
	LMG 6618 (= CCUG 12017)	bv. fecalis	Ovine faeces, Toronto, Canada
	LMG 6617 (= CCUG 12015)	bv. fecalis	Ovine faeces, Toronto, Canada
	LMG 8536 (= CCUG 22580)	bv. fecalis	Ovine faeces, Montana, USA
	LMG 8847 ^T (= NCTC 11366 ^T)	Penner serotype 4	Porcine faeces, Brussels, Belgium
	LMG 7788 ^T (= NCTC 11485 ^T)		Human gingival sulcus, Boston, USA
	LMG 7609 ^T (= NCTC 11649 ^T)		Human, alveolar abscess, Virginia, USA
	LMG 8849 ^T (= NCTC 10842 ^T)		Ovine, foetus brain, Paris, France
	LMG 6443 ^T (= NCTC 10354 ^T)		Bovine, heifer vaginal mucus, Reading, UK
	NCTC 12738 ^T		Human, periodontitis, Boston, USA
<i>C. helveticus</i>	LMG 14731 (= CCUG 13143)		
	LMG 12638 ^T (= NCTC 12470 ^T)		Feline, faeces, Berne, Switzerland
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	LMG 7817 ^T (= NCTC 11608 ^T)		Porcine intestine, proliferative enteritis, Minnesota, USA
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	LMG 14432 ^T (= CHY 5 ^T)		Porcine stomach, UK
<i>C. jejuni</i> subsp. <i>doylei</i>	LMG 8843 ^T (= NCTC 11951 ^T)		Human, infantile diarrhoea, Adelaide, Australia
<i>C. jejuni</i> subsp. <i>jejuni</i>	LMG 8841 ^T (= NCTC 11351 ^T)	Penner serotype 23	Bovine faeces, Brussels, Belgium
<i>C. lari</i>	LMG 8846 ^T (= NCTC 11352 ^T)		Herring gull, cloacal swab, UK
<i>C. mucosalis</i>	LMG 6448 ^T (= NCTC 11000 ^T)	Serotype A	Porcine, small intestine, porcine intestinal adenomatosis, Edinburgh, UK
<i>C. rectus</i>	LMG 7613 ^T (= NCTC 11489 ^T)		Human periodontal pocket, Boston, USA
<i>C. showae</i>	LMG 12635 ^T (= CCUG 30254 ^T)		Human, gingival crevice, Showa, Japan
<i>C. upsaliensis</i>	LMG 8850 ^T (= NCTC 11541 ^T)		Canine faeces, Malmo, Sweden
Related species			
<i>Arcobacter nitrofigilis</i>	LMG 7604 ^T (= CCUG 15893 ^T)		Roots of <i>Spartina alterniflora</i> , Canada
[<i>Bacteroides</i>] <i>ureolyticus</i>	LMG 6451 ^T (= NCTC 10941 ^T)		Human, amniotic fluid, Edmonton, Canada
<i>Campylobacter</i> -like free-living	CCUG 13942		Anaerobic sludge, Netherlands
<i>Helicobacter pylori</i>	LMG 7539 ^T (= NCTC 11637 ^T)		Human gastric antrum, gastritis, Australia

Campylobacters are considered the most common bacterial cause of human enteritis worldwide (12, 37). Although most cases of campylobacter enteritis in man are attributed to the taxa *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis* (12, 37), it has been suggested that the importance of other species also associated with gastrointestinal illness may be significantly underdiagnosed as a consequence of inappropriate isolation and identification methods (6, 20). Several reports support this view (16, 18). As part of an investigation concerning the prevalence, and potential health risk of infrequently encountered campylobacteria in common food animals, an effective procedure for isolating a broad range of species (2) was used to examine the rectal contents of healthy cattle (1). During this investigation, 44 strains, which could not be assigned to any known species of *Campylobacter*, *Arcobacter* or *Helicobacter*, were cultured. These strains were referred to as the catalase-negative, urease-positive campylobacter (CNUPC) group, according to their most distinctive biochemical features.

The aim of the present investigation was to classify 14 representative strains of the CNUPC group by a polyphasic taxonomic approach. Our results show that these isolates represent a previously unreported *C. sputorum* variant, for which the name *C. sputorum* by. *paraureolyticus* is proposed; an additional strain from human enteritis is identified. We also present additional data demonstrating that the present criteria for delineating the named biovars of *C. sputorum* (*sputorum*, *bubulus* and *fecalis*) should be reviewed. We propose a revised infrasubspecific division of this species, to include the biovars *sputorum*, *fecalis* and *paraureolyticus*. The species description of *C. sputorum* is emended accordingly.

METHODS

Bacterial strains and growth conditions. The bacterial strains used, together with their former and present names (where appropriate) and their sources (where known), are listed in Table 1. These included 15 strains assigned to the CNUPC group [(1) and below] and 15 genetically identified (3, 33, 39; P. Vandamme, unpublished data) type and reference strains of *C. sputorum*, representing the biovars *sputorum*, *fecalis* and *bubulus*. In addition, 21 type or reference strains representing the remaining species and subspecies of *Campylobacter*, and the related taxa *Arcobacter nitrofigilis*, [*Bacteroides*] *ureolyticus*, *Helicobacter pylori* and the unnamed free-living *Campylobacter*-like species of Laanbroek *et al.* (15), were included as study references. Strains were cultured for phenotypic characterization (24), whole-cell protein analysis (44), DNA–DNA hybridization (44) and PCR amplification of 16S rDNA (24) according to previously described methods.

Phenotypic characterization. Thirty-nine phenotypic characters were recorded for each of the 51 strains listed in Table 1. Tests were performed using the recommended media and previously described methods (14, 22–26). However, nalidixic acid, cephalothin, metronidazole and carbenicillin-containing media were prepared using filter-sterilized sol-

utions prepared from the native antibiotic (Sigma). All tests were performed on two separate occasions, on freshly prepared media, the quality of which had been tested using appropriate control strains (S. L. W. On, unpublished data).

Numerical analysis of phenotypic tests. Procedures described previously were used (26) with one modification: results for hydrogen sulfide production in triple sugar iron agar (H₂S/TSI) were not graded according to reaction intensity but recorded as positive or negative only, to account for results obtained with LMG 11761 (see below). Briefly, the simple matching coefficient was employed to calculate similarities between strains and a dendrogram was constructed to reflect these similarities. Strains were clustered by the unweighted pair group method with arithmetic averages (UPGMA). All analyses were performed using the computer program MVSP (Multi-Variate Statistical Package, version 2.01L, Kovach Computing Services, Anglesey, UK).

Extraction, separation and analysis of bacterial whole-cell proteins. Protein samples were prepared, separated by PAGE, digitized and subjected to comparative numerical analysis as described previously (31), except that points 6–107 and 141–292 of the 400 points comprising each interpolated trace were used in the analysis. The theoretical and practical reasons for this process have been discussed previously (31).

Preparation of DNA. Native DNA was prepared for DNA–DNA hybridization (44), and PCR amplification of 16S rDNA (using a phenol/chloroform extraction procedure) (35), as described previously.

DNA–DNA hybridization experiments. The degree of DNA–DNA binding, expressed as a percentage, was determined spectrophotometrically by the initial renaturation rate method of De Ley *et al.* (8). Each value given was the mean of at least two hybridization experiments. Values of 30% DNA binding and less do not represent significant DNA homology with the method used. The total DNA concentration was 39 µg ml⁻¹, and the optimal renaturation temperature in 2 × SSC (sodium saline citrate; 1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) was 64.6 °C.

Amplification, sequencing and analysis of 16S rDNA. PCR amplification of 16S rDNA was performed using the 27F/1392r primer pair (Table 2). The final reaction mixture (50 µl) contained 1 µl genomic DNA (~100 ng), 0.25 µM each primer, 0.2 mM dNTPs, 2.5 mM MgCl₂, 0.5 U *Taq* DNA polymerase (Perkin-Elmer) and 1 × PCR buffer (Perkin-Elmer). After an initial denaturation step (94 °C for 5 min), the samples were subjected to 30 amplification cycles comprising denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 1 min), completed by a prolonged extension step (72 °C for 7 min). After checking the integrity of the PCR product by agarose gel electrophoresis, amplified DNA was purified for sequencing using a QIAquick PCR purification kit according to the manufacturer's recommendations (Qiagen). Sequences were determined directly using a dye terminator cycle sequencing kit under conditions recommended by the manufacturer (Applied Biosystems) using the eight primers listed in Table 2. Sequencing reactions were run on an Applied Biosystems model 373A automated DNA sequencer, using the manufacturer's recommendations. The percentage similarity between the sequence determined for CNUPC strain BU 112B (GenBank accession no. AF022768), and all other strains contained in the GenBank database, was determined

Table 2. PCR primers used for amplification and sequencing of 16S rDNA

Conventional nomenclature of primers is used here; the number given is the number of the *E. coli* base position to which the 3'-end of the listed primer anneals: f, forward; r, reverse. Single-letter codes are standard International Union of Biochemistry codes for bases and ambiguity.

Primer	Sequence (5' → 3')
27f	AGA GTT TGA TCM TGG CTC AG
518r	GTA TTA CCG CGG CTG CTG G
536f	CAG CAG CCG CGG TAA TAC
787r	GGA CTA CHA GGG TAT CTA AT
806f	ATT AGA TAC CCT DGT AGT CC
1054r	ACG AGC TGA CGA CRR CCA TG
1073f	TGG TTG TCG TCA GCT CGT
1392r	ACG GGC GGT GTG TRC

using the FASTA program (30) provided as part of the GCG package (10).

RESULTS

Phenotypic characterization

All of the 30 *C. sputorum* strains studied (including members of the CNUPC group) were Gram-negative 'S'-shaped and/or curved rods when examined under microscopy. Phenotypic variation between strains was minimal; a detailed report of the species' phenotype is given below in the emended description. Reproducible differences in six tests allowed isolates to be divided into seven biochemical variants (described below). One variation noted was in the production of H₂S in TSI medium. Most (28/30) of the *C. sputorum* strains examined consistently produced copious quantities of H₂S in TSI. However, LMG 11761 yielded copious and trace amounts of H₂S in TSI medium, respectively, on the two occasions it was tested. In addition, LMG 14261 gave a negative result on both occasions. Consequently, the H₂S/TSI test was recorded only as positive or negative and was not scored according to intensity of reaction, as done previously (21, 26).

Several tolerance tests gave results which were not wholly reproducible and could not be used to define variation. These tests, and the corresponding percentage of strains giving a reproducible result (in parentheses), were growth on: 1.0 (45%), 1.5 (96%) and 2.0% (59%) bile; 3.5 (86%) and 4.0% (83%) sodium chloride; and 0.02% safranin (69%). Nonetheless, all *C. sputorum* bv. *sputorum*, *C. sputorum* bv. *fecalis* and CNUPC group strains could be distinguished from each other and from other *Campylobacter* species on the basis of their phenotypic characteristics (Table 3).

Numerical analysis of phenotypic tests

Only those phenotypic characters yielding fully reproducible results were used in the numerical analysis. Examination of the dendrogram produced (Fig. 1) showed that 18 phenons were formed at the 89% similarity (S-) level. Thirteen phenons comprised single strains included as study references, whilst strains of *Campylobacter gracilis*, *Campylobacter hyointestinalis* (both subspecies) and *C. fetus* (both subspecies) were also contained in discrete clusters. The remaining two phenons respectively included the closely related catalase-negative enteropathogenic species *Campylobacter upsaliensis* and *Campylobacter helveticus*; and 30 strains of *C. sputorum*, including 15 strains assigned to the CNUPC group. Furthermore, within the *C. sputorum* cluster, seven subphenons were formed at the 98% S-level, each defined by a particular phenotype. Subphenon I contained the type strain of *C. sputorum* and five other catalase-negative isolates of human, bovine, ovine and porcine origin, whilst subphenon II comprised strains of ovine and bovine fecal origin that were catalase-positive (bv. *fecalis*). Subphenon III was a single ovine fecal strain differing from those of subphenon II only in its inability to reduce nitrate. Subphenon IV contained an ovine strain resembling subphenon I, but resistant to metronidazole (4 mg l⁻¹) when tested on a nutrient agar base. Subphenon V contained 13 cattle isolates originally classified as the CNUPC group (and therefore catalase-negative, urease-positive), in addition to a human diarrhoeal isolate (LMG 11764). Subphenon VI comprised a single cattle CNUPC isolate differing from the other strains of this phenotype only in its tolerance to cephalothin (32 mg l⁻¹). Subphenon VII contained a catalase-negative strain isolated from human pus, unique in that the H₂S/TSI test was negative.

Numerical analysis of whole-cell protein profiles

Forty strains were used in the analysis, including five CNUPC strains representing each of the three genotypes determined by macrorestriction profiling (1). A further 16 *C. sputorum* strains were included, in addition to 19 type and reference strains of other taxa. The dendrogram produced (Fig. 2) clearly identified the CNUPC group as highly related to a human strain also giving the CNUPC phenotype (LMG 11764), and clustered these strains with 13 other *C. sputorum* strains at the 69% S-level, clearly separating these strains from all other taxa included. The aberrant position of two *C. sputorum* strains (LMG 8535 and LMG 10388) in the dendrogram was attributed to the presence of a high-molecular-mass dense protein band in these strains, since their profiles were otherwise typical of the other *C. sputorum* strains studied (data not shown).

The main *C. sputorum* cluster could be further subdivided into subphenons at higher S-levels, but no single cut-off level could delineate these strains into groups corresponding with either their source, or the

Table 3. Characteristics differentiating *C. sputorum* biovars from other campylobacteria

Phenotypic data on *C. sputorum* were as described in this study. The characteristics of the other taxa are from references (27), (29) and (45) and were determined with the same methods. +, All strains positive; -, all strains negative; (+), 70–90% strains positive; (–), 7–29% strains positive; v, 40–64% strains positive. Abbreviations: Cat, catalase; Ure, urease; APho, alkaline phosphatase; Hipp, hippuricase; IA, indoxyl acetase; Gly, glycine; TTC, triphenyltetrazolium chloride; Nal, nalidixic acid.

	Production of:						Nitrate reduction	Tolerance to:			
	Cat	Ure	APho	Hipp	IA	H ₂ S/TSI*		2.0% NaCl	1% Gly	TTC	Nal
<i>C. sputorum</i> bv. paraureolyticus	–	+	–	–	–	+	+	+	+	–	+
bv. nov.											
<i>C. sputorum</i> bv. sputorum	–	–	–	–	–	(+)	+	+	+	–	+
<i>C. sputorum</i> bv. fecalis	+	–	–	–	–	+	(+)	+	+	–	+
<i>C. coli</i> †	+	–	–	–	+	v	+	–	(+)	+	(–)
<i>C. concisus</i>	–	–	(+)	–	–	(–)	(–)	(–)	(–)	–	(+)
<i>C. curvus</i>	–	–	v	(–)	v	(–)	+	v	+	+	+
<i>C. fetus</i> subsp. <i>fetus</i>	+	–	–	–	–	–	+	–	+	–	+
<i>C. fetus</i> subsp. <i>venerealis</i>	(+)	–	–	–	–	–	(+)	–	(–)	–	(+)
<i>C. gracilis</i>	(–)	–	–	–	(+)	–	(+)	(–)	+	–	v
<i>C. helveticus</i>	–	–	–	–	+	–	+	(–)	v	–	–
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	+	–	–	–	–	(+)	+	–	+	(–)	+
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	+	–	(–)	–	–	(+)	+	–	(–)	–	+
<i>C. jejuni</i> subsp. <i>doylei</i>	(+)	–	–	+	+	–	–	–	(–)	v	–
<i>C. jejuni</i> subsp. <i>jejuni</i>	+	–	–	+	+	–	+	–	(+)	(+)	–
<i>C. lari</i>	+	v	(–)	–	(–)	–	+	(+)	+	(+)	(–)
<i>C. mucosalis</i>	–	–	(+)	–	–	+	(–)	(+)	v	–	(+)
<i>C. rectus</i>	(–)	–	–	–	+	–	+	v	+	–	(+)
<i>C. showae</i>	+	–	–	–	v	v	+	+	v	–	–
<i>Campylobacter</i> sp., free-living	–	–	–	–	+	–	+	–	–	+	–
<i>C. upsaliensis</i>	–	–	–	–	+	–	+	–	+	v	–
<i>A. nitrofigilis</i>	+	+	–	–	+	–	+	+	–	–	–
[<i>B.</i>] <i>ureolyticus</i>	(–)	+	–	–	(–)	–	+	+	+	–	–
<i>H. pylori</i>	+	+	+	–	(–)	–	–	–	–	v	(+)

* Strains of *C. sputorum* and *C. hyointestinalis* subsp. *lawsonii* normally produce copious amounts of H₂S on TSI agar slopes [but see text and (29)].

† Includes data on *C. coli* strains previously described as *C. hyoilei*; see (45).

full extent of the biochemical variation noted here (see above). However, at the 84% S-level, three subphenons were evident. Subphenon a contained 12 urease-negative strains, whilst subphenon b consisted of six urease-positive isolates. Subphenon c comprised LMG 14261, a strain unique in its inability to produce hydrogen sulfide.

DNA–DNA hybridizations

Six representative strains of *C. sputorum* (including the human strain assigned to the CNUPC group) and type strains of four other *Campylobacter* spp., were chosen for DNA homology studies. DNA binding values ranging from 82–96% ($\pm 6\%$) were obtained between strains of *C. sputorum*, whilst comparable values between *C. sputorum* and each of the type strains of

four distinct *Campylobacter* spp. were $\leq 22\%$ (Table 4).

PCR amplification and sequence analysis of 16S rDNA

The size of the 16S rDNA PCR product of cattle isolate BU 112B obtained by use of primers 27f and 1392r was estimated as approximately 1650 bp, some 250 bp larger than that expected for other *Campylobacter* spp., but equivalent to that expected for *C. sputorum* due to the presence of an internal transcribed spacer (40). The sequencing primers used (Table 2) enabled a sequence of 1561 bases of the rRNA gene to be determined (representing approx. 90% of the rRNA gene); the sequence of the first 200 bases was resolved in the forward direction only. Nucleotide sequence similarity searching of the GenBank database with the program FASTA (30) identified the most closely related

Fig. 1. Dendrogram of the cluster analysis based on phenotypic data of strains examined. The numbers on the horizontal axis indicate the percentage similarities as determined by the simple matching coefficient and UPGMA clustering. Phenons were formed at the 89% similarity (S-) level, with subphenons of *C. sputorum* (indicated by roman numerals) delineated at the 98% S-level.

sequence to BU 112B (accession no. AFO22768) as that of the 16S rRNA gene sequence of the type strain of *C. sputorum* (LMG 7795^T; accession no. X67775)

(40), with which it showed 99.7% similarity. Of the five base differences, four were found to occur within the internal transcribed spacer region (Fig. 3).

Fig. 2. Dendrogram of the cluster analysis based on partial protein profiles of strains examined. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient and UPGMA clustering. Phenons were formed at the 69% S-level, with subphenons of the principal *C. sputorum* cluster (indicated by lower-case letters) delineated at the 84% S-level.

Table 4. DNA-DNA hybridization values of *C. sputorum* strains

<i>C. sputorum</i> biovar	Strain no.	<i>Campylobacter</i> reference strains:					
		<i>C. sputorum</i> bv. <i>sputorum</i> LMG 7795 ^T	<i>C. sputorum</i> bv. <i>fecalis</i> LMG 6617	<i>C. coli</i> LMG 8847 ^T	<i>C. concisus</i> LMG 7788 ^T	<i>C. fetus</i> subsp. <i>fetus</i> LMG 8849 ^T	<i>C. jejuni</i> subsp. <i>jejuni</i> LMG 8841 ^T
paraureolyticus	LMG 11764	89					
sputorum	LMG 7975		83				
sputorum	LMG 11761	89					
sputorum	LMG 11765	92					
fecalis	LMG 6617	82	100		7	14	22
fecalis	LMG 6618		96	21			

DISCUSSION

Classification of the CNUPC group as *C. sputorum*, identification of a human isolate, and proposal of *C. sputorum* bv. *paraureolyticus*

Numerical analyses of both phenotypic tests and protein profiles clearly indicated the CNUPC group to be a homogeneous taxon, and also assigned a human

strain, LMG 11764 (not originally included in the original study group from cattle faeces) to this group. Moreover, the CNUPC group was shown to be highly related to type and reference strains of *C. sputorum* in each analysis. The concordance of numerical analysis of protein profiles with DNA-DNA hybridization data is well-known (21, 31, 44-45) and was again confirmed in this study, where the genomic relatedness


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X67775      255-AAGCTTTATATTAATAAAGCGAAAAAAGC-285
          *      *      *      *
AFO22768    247-CAGCTTTATA.TAATAAAGCG.AAAAAAGT-275

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Fig. 3. Alignment of a 31 bp region of the 16S rRNA gene insertion sequences of X67775 (*C. sputorum* bv. *sputorum*, LMG 7795^T) (40) with the corresponding region of AFO22768 (*C. sputorum* bv. *paraureolyticus*, BU 112B), summarizing the differences (asterisks) (including alignment gaps indicated by full stops) between the two sequences. Bases are numbered according to sequences submitted to GenBank.

of the human CNUPC strain to selected *C. sputorum* isolates was shown to be well within the range expected for a single species (47) (Table 4). It is also noteworthy that LMG 11764 was identified as a *C. sputorum* isolate by use of a species-specific PCR assay based on a variable region of the 23S rRNA gene (3), but no phenotypic data were presented by these authors. Furthermore, the 16S rDNA sequence of a typical bovine CNUPC strain (LMG 17590) was 99.7% similar to that of the type strain of *C. sputorum* and contained an insert of about 250 bp, a feature characteristic of this species (40). Although urease activity is considered an unusual feature among other taxa belonging to the *Campylobacteraceae* (41), these data clearly identify the CNUPC group as a novel biovar for which we propose the name *C. sputorum* bv. *paraureolyticus* (formal description is given below).

Re-evaluation of the existing infrasubspecific (biovar) divisions of *C. sputorum*

The original concept of the infrasubspecific division of this species was based on one fundamental principle: that certain biochemical characteristics could be used to differentiate *C. sputorum* strains isolated from particular sources and believed to be host-specific (19, 33). Strains from the human oral cavity (bv. *sputorum*), the bovine and ovine genital tract (bv. *bubulus*), and ovine faeces (bv. *fecalis*) are traditionally differentiated by their catalase reaction (only bv. *fecalis* produces catalase) and tolerance to 3.5% NaCl and 1% bile (bv. *sputorum* giving a negative and positive result, respectively, in these two tests) (33). However, the validity of this infrasubspecific division has been questioned, since the results of tolerance tests using various concentrations of NaCl and ox bile are not reproducible (22–23, this study). The continued use of tolerance to NaCl and ox bile as determinative phenotypic markers for biovars of *C. sputorum* strains isolated from differing sources is clearly unjustified. Although tolerance to 0.02% safranin was shown to be a potential marker for certain bovine strains of *C. sputorum* (22), our data show that CNUPC strains do not give reproducible results in the latter test (see above). Indeed, no phenotypic traits unequivocally differentiate catalase- and urease-negative strains from human, bovine, porcine and ovine sources (22, this study).

Although tolerance test results are inappropriate for

defining 'source-specific biovars' of *C. sputorum*, it is equally clear that a useful infrasubspecific division of the species can be achieved by the determination of catalase and urease activity. The validity of such a division is strongly supported by the correlation between the aforementioned enzyme activities and the results of numerical analyses of whole-cell protein profiles of *C. sputorum*, as determined in this study (for urease-positive strains) and a previous investigation (for catalase-positive and -negative strains) (22). Moreover, catalase and urease tests are extremely reliable when used to characterize campylobacteria (25), and this feature is clearly important in considering the definition of a biovar. The additional phenotypic diversity observed among *C. sputorum* strains examined here (in nitrate, H₂S/TSI, cephalothin and metronidazole tests) may eventually lead to the definition of additional biovars, but at present the limited number of strains showing these phenotypes make such proposals inappropriate. We conclude that three named biovars of *C. sputorum* should be recognized according to their ability to produce catalase and urease: bv. *sputorum* (negative in both tests), bv. *fecalis* (produces catalase only) and bv. *paraureolyticus* (produces urease only). We recommend that strains previously assigned to bv. *bubulus* be re-designated as bv. *sputorum*, unless phenotypic or pathological properties which reliably differentiate catalase-negative strains of bovine, porcine, ovine and human origin can be identified.

C. sputorum is largely regarded as a commensal in the hosts in which it is found (33, 37). However, cases of enteritis (18, 33; this study), abscess formation and other skin lesions (4, 28, 33, this study) and bronchitis (38) in humans have been reported. Moreover, both *C. sputorum* bv. *paraureolyticus* and bv. *sputorum* have been isolated from food animals and human infectious material (4, 18, 28, 33; this study). The use of sensitive isolation (6) and accurate identification procedures (20) are thus required to properly determine the role of *C. sputorum* in human disease and consequently its zoonotic potential.

Emended description of *Campylobacter sputorum* Véron and Chatelain 1973

Cells are non-spore-forming, Gram-negative rods (0.3–0.5 µm wide and 2–4 µm long) which are curved or loosely helical ('S'-shaped). Motile by means of a single, unsheathed, polar flagellum. Glucose is not fermented or oxidized. Optimal growth occurs under microaerobic (about 5% O₂) conditions, but strains will also grow anaerobically, whether or not trimethylamine-*N*-oxide (TMAO, 0.1%) is present in the growth medium. No growth occurs in air or at room temperature (18–22 °C) under microaerobic conditions. After 72 h growth on 5% blood agar at 37 °C, colonies are circular, convex, and 1.0–2.0 mm in diameter. Pitting of the agar around the area of bacterial growth is not seen. A greenish colouration,

accompanied by α -haemolytic activity, is often observed, although this feature is inconsistent for some strains. Oxidase is produced. Most strains reduce nitrate. Hydrolysis of alkaline phosphatase, DNA, hippurate and indoxyl acetate is not observed. Triphenyltetrazolium chloride (TTC) is not reduced. Most strains produce copious quantities of H_2S in TSI medium, but occasionally a weaker or even negative result may be observed. Strains grow in the presence of 1% glycine, 2% NaCl, nalidixic acid (32 mg l^{-1}), and 0.032% methyl orange: a few strains demonstrate tolerance to metronidazole (4 mg l^{-1} , using a nutrient agar base) or cephalothin (32 mg l^{-1} , blood agar base). Strains do not grow in the presence of 0.04% TTC, 0.1% $KMnO_4$, or pyronin (0.02%, nutrient and blood agar base media). The G+C content of the DNA from *C. sputorum* is 30–33 mol% (33, 43). The pathogenic potential of *C. sputorum* is unknown.

Strains of *C. sputorum* may be assigned to one of three biovars according to their reaction in two tests, catalase and urease. *Campylobacter sputorum* bv. *sputorum* comprises catalase- and urease negative strains that have been found in the oral cavity, faeces (normal and diarrhoeic) and abscesses (and other skin lesions) of humans; the genital tract of bulls; abortions in sheep; and the faeces of sheep and pigs. *Campylobacter sputorum* bv. *fecalis* comprises catalase-positive, urease-negative strains that have been isolated from ovine and bovine faeces. LMG 8531 is recommended as a reference strain.

Description of *Campylobacter sputorum* bv. *paraureolyticus* bv. nov.

Campylobacter sputorum bv. *paraureolyticus* bv. nov. (para.ureo.lyt'ic.us. Gr. prep. *para* alongside of, resembling; M.L. *ureolyticus* specific epithet, *paraureolyticus* resembling *Bacteroides ureolyticus*, a urease-producing bacterium closely related to other *Campylobacter* spp.).

Comprises catalase-negative, urease-positive strains isolated from human diarrhoea and bovine faeces. LMG 11764 (human) and LMG 17590 (= CCUG 37579) (bovine) are recommended reference strains. The type strain of *C. sputorum* belongs to bv. *sputorum* and is LMG 7795^T (= CCUG 9728 = NCTC 11528 = ATCC 35980) and shows phenotypic properties typical of the species (see above). This strain was isolated from the human oral cavity. The G+C content of its DNA is 31 (43) to 32 mol% (33).

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