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Clonal Nature of *Campylobacter fetus* as Defined by Multilocus Sequence Typing

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Campylobacter fetus **can be divided into the subspecies** ***C. fetus*** **subsp.** ***fetus*** **and** ***C. fetus*** **subsp.** ***venerealis*****.** ***C. fetus*** **subsp.** ***fetus*** **causes sporadic infections in humans and abortion in cattle and sheep and has been isolated from a variety of sites in different hosts.** ***C. fetus*** **subsp.** ***venerealis*** **is host restricted, being isolated mainly from the genital tracts of cattle, and is the causative agent of bovine genital campylobacteriosis. Despite differences in niche preference, microbiological subspecies differentiation has proven difficult. Different typing methods divided** ***C. fetus*** **isolates into different subgroups, depending on the methods used. The relative value of these methods can be assessed by the evolutionary relationship of isolates belonging to the genus; therefore, we developed a multilocus sequence typing (MLST) scheme for** ***C. fetus*****. This scheme was applied to 140** ***C. fetus*** **isolates previously typed by amplified fragment length polymorphism (AFLP) analysis. A total of 14 different sequence types (STs) were identified, and these exhibited low levels of inter-ST genetic diversity, with only 22 variable sites in 3,312 nucleotides. These MLST data indicate that** ***C. fetus*** **is genetically homogeneous compared to the homogeneity of other** ***Campylobacter*** **species. The two** ***C. fetus*** **subspecies were extremely closely related genetically, but ST-4 was associated only with** ***C. fetus*** **subsp.** ***venerealis*****, which represents a "bovine" clone. The** ***C. fetus*** **subsp.** ***fetus*** **isolates studied were more diverse in terms of their STs, and the STs correlated with epidemiological relationships. Congruence was observed among** ***C. fetus*** **subspecies,** ***sap*** **type, and ST; therefore, MLST confirms that mammalian** ***C. fetus*** **is genetically stable, probably as result of the introduction of a single ancestral clone into a mammalian niche.**

Campylobacter fetus is currently divided into the two subspecies *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (38). *C. fetus* subsp. *venerealis* also includes a phenotypic biovar designated *C. fetus* subsp. *venerealis* bv. intermedius (8, 12). Both subspecies are primary pathogens; *C. fetus* subsp. *fetus* is associated with abortions most often in sheep and to a lesser extent in cattle and humans and can frequently be isolated from the gallbladders and intestinal tracts of a variety of hosts, including ungulates, fowl, reptiles, and humans (12). *C. fetus* subsp. *venerealis* is the causative agent of bovine genital campylobacteriosis, an infectious venereal disease which may lead to reproductive problems, such as sterility and abortion. *C. fetus* subsp. *venerealis* is host restricted, being isolated primarily from the bovine genital tract (12, 38), whereas *C. fetus* subsp. *fetus* can be isolated from a wider range of species.

The subspecies are conventionally defined with biochemical tests, namely, growth in 1% glycine (32) and H_2S production from medium containing 0.02% cysteine (1). Despite these differences and host and niche preferences, studies that have used potentially discriminatory methods, including DNA-DNA hybridization (29, 37), serotyping (27), ribotyping (3), cellular fatty acid analysis (2), and whole-cell protein analysis (37), have been unable to distinguish *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Therefore, from a taxonomic viewpoint it is questionable whether *C. fetus* should be divided into subspecies. However, a dichotomy has been detected among *C. fetus* serotype A and B strains (7, 22, 27). These serotypes reflect differences in both lipopolysaccharide structure and the type of surface layer protein (SLP) and surface array protein (Sap). The two serotypes correlate with the corresponding *sap* type, *sapA* or *sapB* (33). *C. fetus* subsp. *venerealis* strains are type A, whereas *C. fetus* subsp. *fetus* strains can be either serotype A or serotype B.

Several molecular methods, including pulsed-field gel electrophoresis (PFGE) fingerprinting (25, 31), amplified fragment length polymorphism (AFLP) analysis (39), and PCR amplification (15, 40), have been used for subspecies differentiation; but they give contradictory results. Recently, subspecies-specific random amplification of polymorphic DNA PCRs were described, but they were evaluated with a limited number of strains (34). A new *C. fetus* subsp. *venerealis*-specific PCR has been developed based on an AFLP marker (36). Subspecies differentiation is of such statutory importance that additional

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unambiguous typing methods are still required, preferably those that assist with epidemiological investigations. This may be useful for outbreak control and has been performed by using serotyping and PFGE. The currently available serotyping scheme has a low level of discrimination, as only a few lipopolysaccharide serotypes have been detected. However, PFGE has proven discriminatory and has been applied in epidemiological studies of both human and veterinary infections (11, 20, 23, 24, 28).

Multilocus sequence typing (MLST) is a nucleotide sequence-based approach to bacterial typing in which variations in ~500-bp fragments of housekeeping genes (generally seven) are indexed. MLST is useful for studies of bacterial epidemiology and population genetics, including studies of species of the genus *Campylobacter* (4, 5, 9, 18, 21). MLST has the advantages that variation in the target gene is accessed directly and that the technology employed is both readily disseminated and highly reproducible among laboratories (19). Furthermore, comparisons of the population structures and population genetics of different species can be made by using MLST data, particularly if the same loci are used for different species.

Here we describe an MLST scheme for *C. fetus* that uses the same loci as a previous scheme for *C. jejuni* and *C. coli* (4, 5). We tested this scheme using a diverse collection of isolates and examined the *C. fetus* population structure and genetic diversity, together with the suitability of MLST for studies of *C. fetus* subspecies differentiation and epidemiology.

MATERIALS AND METHODS

Bacterial strains. A collection of 140 *C. fetus* isolates, including 8 reference strains and 132 field isolates (Table 1) were included. This diverse collection contained both epidemiologically unrelated isolates, and isolates from seven outbreaks which occurred at bovine artificial insemination (AI) stations. Isolates were obtained from bovine, ovine, human, equine, and monkey hosts located in different geographical areas: Argentina, Belgium, France, Germany, Hungary, Japan, The Netherlands, South Africa, Spain, Turkey, the United Kingdom, and the United States (Table 1). Isolates were grown under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2, 80% N_2; Anoxomat; Mart Microbiology B.V., Lichtenvoorde, The Netherlands) on heart infusion agar supplemented with 5% sheep blood (Biotrading, Mijdrecht, The Netherlands) for 3 days and were stored at −80°C in heart infusion broth containing 15% glycerol.

Preparation of chromosomal DNA. Chromosomal DNA was isolated by using the Puregene kit (Gentra Systems, Minneapolis, MN) and diluted to a final concentration of approximately 5 ng/μl for AFLP analysis and PCR and to 25 ng/μl for PCR amplification during MLST.

Species identification and subspecies differentiation. Isolates were identified genotypically as *C. fetus* by using a previously described PCR protocol (15), with modifications (39). The subspecies were then identified genotypically by Cf C05 PCR (36); the PCR of Hum et al. (15); AFLP analysis (39); and biochemically, according to growth in the presence of 1% glycine (Merck, Darmstadt, Germany) (32) and H_2S production from brucella broth (Difco, Becton Dickinson, Franklin Lakes, NJ) containing 0.02% cysteine (Sigma Aldrich, St. Louis, MO) (1, 17).

***sap* typing.** *sap* typing was performed with PCR primers based on the *sap*A and *sap*B 5′ conserved regions, as described by Tu et al. (33).

AFLP analysis. AFLP analysis was performed by a previous method, with modifications (6, 39). These enhancements comprised the use of a capillary-based sequencing system (ABI 3100; Applied Biosystems, Foster City, CA) and data analysis with Bionumerics 3.5 software (Applied Maths, St-Martens-Latem, Belgium). Chromosomal DNA was digested with the restriction enzymes HindIII and HhaI, and site-specific adaptors were ligated. A preselective PCR amplification was performed, followed by selective PCR with primers containing a 3′ selective nucleotide and one fluorescently labeled (6-carboxyfluorescein) primer. PCR products underwent electrophoresis through an ABI 3100 capillary sequence system by using a performance optimized polymer-4 polymer matrix (Applied Biosystems). AFLP patterns were analyzed by importing the AFLP data (via the conversion software GeneScan 3.7 [Applied Maths]) into the Bionumerics 3.5 software. Patterns were normalized by referring to the molecular mass of the internal DNA marker (GeneScan-500 [carboxy X rhodamine]; Applied Biosystems). The genetic similarity between the patterns was calculated by using the Pearson product-moment correlation coefficient, and clustering analysis was performed by the unweighted pair group method with arithmetic averages for the region corresponding to 84.6% to 87.1% of the band pattern.

PFGE. PFGE was performed as described previously by using the restriction enzymes SmaI and SalI (36a)

Multilocus sequence typing. (i) Primer design. *C. fetus* sequences corresponding to the seven loci used in a previously described *C. jejuni* and *C. coli* MLST scheme were required: *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA* (4, 5). Various *C. jejuni* and *C. coli* MLST primers (Sigma-Genosys, Haverhill, United Kingdom) were employed in multiple combinations to identify primers that would amplify these sequences from *C. fetus*. A reduced annealing temperature of 48°C was used to improve the binding of the *C. jejuni* primers to heterologous *C. fetus* DNA. Of the many primer combinations tested, those that provided amplification products were as follows: for *C. jejuni aspA*, primers A9 and A10; for *C. jejuni glnA*, primers A1 and A2; for *C. jejuni gltA*, primers A1 and A2; for *C. jejuni glyA*, primers A1 and A2; for *C. jejuni tkt*, primers A5 and A4; and for *C. coli uncA*, primers A1 and A2 (the sequences of all primers except *C. jejuni tkt* A5 and *C. coli uncA* A1 and A2 are available at http://pubmlst.org/campylobacter/; the sequence of *tkt* A5 is 5′-TTTAAGTGCTGATATGGTGC-3′, the sequence of *C. coli uncA* A1 is 5′-ATGTAGCCATAGGACAAAAGC-3′, and the sequence of *C. coli uncA* A2 is 5′-CATTCTTGTCGCGTTCAGTTG-3′). The *C. fetus* sequence corresponding to the *pgm* locus failed to amplify with any *C. jejuni*- or *C. coli*-specific primers; however, this sequence was kindly provided by D. Sanchez, Universidad Nacional de General San Martín, Instituto de Investigaciones Biotecnológicas, San Martín, Argentina. The *C. fetus* amplicons were sequenced directly by using the same primers used for amplification. Nucleotide sequence extension reactions were carried out by using BigDye ready reaction mix (version 3; Applied Biosystems), in accordance with the manufacturer's instructions. The reaction products were separated with an ABI 3730 automated DNA sequencer (Applied Biosystems).

These *C. fetus* sequences from the seven loci were used to design seven pairs of *C. fetus*-specific primers for MLST (Table 2). The MLST allele trimming sites for *C. fetus* were chosen to correspond to the previous *C. jejuni* and *C. coli* sites to assist with future phylogenetic comparisons among species. At least 28 nucleotides separated the MLST trimming site and the 3′ end of the *C. fetus* primers. MLST was performed by using the same primer pair used for both amplification and sequencing.

(ii) PCR amplification and nucleotide sequencing. Seven PCR amplicons were obtained for each isolate by using the primers shown in Table 2. Each 50-μl PCR mixture contained 39.75 μl molecular biology-grade water (Sigma Aldrich), 5 μl 10× PCR buffer (QIAGEN, Crawley, United Kingdom), 1 μl 10 μM each forward and reverse primer, 1 μl 10 mM deoxynucleoside triphosphate mixture (Invitrogen, Paisley, United Kingdom), 0.25 μl HotStar *Taq* DNA polymerase (QIAGEN), and 2 μl *C. fetus* chromosomal DNA (approximately 50 ng). The amplification conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min and storage at 4°C. Nucleotide sequencing (forward and reverse) was performed with the same primers (10 μM diluted 1:15 in water); and the reaction conditions were 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min.

(iii) Allele and ST assignment. For each locus, distinct allele sequences were assigned arbitrary allele numbers in the order of identification. Each genotype was therefore designated by seven numbers (e.g., 1-3-2-4-1-1-2) that constituted an allelic profile or sequence type (ST; e.g., ST-3). The STs were assigned arbitrary numbers in the order of description. All data for newly described *C. fetus* alleles and STs were deposited in a *Campylobacter fetus* MLST database (http://pubmlst.org/cfetus/). New sequences were assigned allele numbers, and isolates were assigned their STs by interrogating the database. Allele numbers for new sequences and ST numbers for new allelic profiles are available by submission to the database.

(iv) Data analysis. Phylogenetic analysis was performed with MEGA (version 2.1) software, available at http://www.megasoftware.net, by using the concatenated MLST gene sequence fragments for each isolate. Data were also subjected to split decomposition analysis by using SPLITSTREE (version 3.1) software (16).

RESULTS

***C. fetus* genotyping and determination of population structure by MLST.** The collection of 140 *C. fetus* isolates was typed by MLST. The *C. fetus* MLST data were used to (i) assess the

TABLE 1. *C. fetus* strains used for typing and the corresponding test results[a]

Strain	Source	Country[a]	ST	Allele no.							Growth in glycine	H_2S production	Result by PCR of:		Result of AFLP analysis	*sap* type	Outbreak (mo-yr)	PFGE type
				aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*			Hum et al. (15)	Van Bergen et al. (36)				
23	Bovine	NL	1	1	1	1	1	1	1	1	+	+	F	–	F	B	Apr-98	1
492116	Bovine	NL	1	1	1	1	1	1	1	1	+	+	F	–	F	B	Apr-98	
m	Bovine	NL	1	1	1	1	1	1	1	1	+	+	F	–	F	B	Apr-98	1
Iz-2149-80	Bovine	NL	1	1	1	1	1	1	1	1	+	+	F	–	F	B	Feb-98	1
501340	Bovine	NL	1	1	1	1	1	1	1	1	+	+	F	–	F	B	Feb-98	1
110800-1-2	Bovine	NL	2	1	2	2	2	1	1	2	+	+	V	–	F	A	Aug-00	11
110800-21-1	Bovine	NL	2	1	2	2	2	1	1	2	+	+	V	–	F	A	Aug-00	
110800-3-1	Bovine	NL	2	1	2	2	2	1	1	2	+	+	V	–	F	A	Aug-00	
110800-6-1	Bovine	NL	2	1	2	2	2	1	1	2	+	+	V	–	F	A	Aug-00	
280900-19	Bovine	NL	2	1	2	2	2	1	1	2	+	+	V	–	F	A	Aug-00	
Ru-25888-7	Bovine	NL	2	1	2	2	2	1	1	2	+	+	V	–	F	A	Aug-00	11
0194-98	Ovine	UK	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
122	Ovine	TR	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
5.5.42	Ovine	NL	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
5516 (ATCC 33248)	Human	USA	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
601	Ovine	NL	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
64	Ovine	TR	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
68	Ovine	TR	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
BT 10/98	Ovine	UK	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
C036959	Human	UK	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
Ru-17722-2	Bovine	NL	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
Sz-2001-00298	Ovine	NL	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
Sz-739-24	Bovine	NL	2	1	2	2	2	1	1	2	+	+	F	–	F	A		
010500-9	Bovine	NL	3	1	2	2	3	1	2	1	+	+	F	–	F	B	Apr-00	7
Ru-13826	Bovine	NL	3	1	2	2	3	1	2	1	+	+	F	–	F	B	Apr-00	7
938230	Bovine	NL	3	1	2	2	3	1	2	1	+	+	F	–	F	B	Nov-00	7
R37178-5	Bovine	NL	3	1	2	2	3	1	2	1	+	+	F	–	F	B	Nov-00	7
3286 (large colony)	Ovine	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
4114	Human	BE	3	1	2	2	3	1	2	1	+	+	V	–	F	B		
5396/7	?	SA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
5517 (ATCC 33249)	Human	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
84-104	Monkey	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
84-107	Human	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
84-90	Bovine	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
84-91	Human	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
84-94	Human	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
87-364	Equine	USA	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
98/v445	Bovine	UK	3	1	2	2	3	1	2	1	+	+	V	–	F	B		10
BT 8/99	Ovine	UK	3	1	2	2	3	1	2	1	+	+	F	–	F	B		
NCTC 10842 (ATCC 27374)	Ovine	F	3	1	2	2	3	1	2	1	+	+	F	–	F	B		9
00/305	Bovine	ARG	4	1	2	2	2	1	2	1	–	+	V	–	INT	A		
01/165	Bovine	ARG	4	1	2	2	2	1	2	1	+	+	F	–	INT	A		
01/210	Bovine	ARG	4	1	2	2	2	1	2	1	–	+	V	–	INT	A		
01/228	Bovine	ARG	4	1	2	2	2	1	2	1	–	+	V	–	INT	A		
02-298	Bovine	ARG	4	1	2	2	2	1	2	1	–	+	V	–	INT	A		
03/293	Bovine	ARG	4	1	2	2	2	1	2	1	+	+	V	–	INT	A		
03/596	Bovine	ARG	4	1	2	2	2	1	2	1	+	+	F	–	INT	A		
10	?	SA	4	1	2	2	2	1	2	1	–	+	F	–	INT	A		
135	?	SA	4	1	2	2	2	1	2	1	–	+	F	–	INT	A		

136	?	SA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		14
154.4	?	SA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
3292 (ADRI 812)	Bovine	USA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
3293 (ADRI 817)	Bovine	USA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
44.1	?	SA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
8	?	SA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
92-203	Bovine	ARG	4	1	2	2	2	1	2	1	−	+	V	−	INT	A		
94/92	Bovine	ARG	4	1	2	2	2	1	2	1	−	+	V	−	INT	A		
97/532	Bovine	ARG	4	1	2	2	2	1	2	1	+	+	F	−	INT	A		
98/25	Bovine	ARG	4	1	2	2	2	1	2	1	−	+	V	−	INT	A		
98/472	Bovine	ARG	4	1	2	2	2	1	2	1	−	+	V	−	INT	A		
98/v156	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		3
98/v444	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
98/v635	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
99/541 gray	Bovine	ARG	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
99/541 white	Bovine	ARG	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
BT 174/03	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
BT 28/98	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
BT 36/98	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
C1N3	Bovine	ARG	4	1	2	2	2	1	2	1	+	+	V	−	INT	A		
v51/99	Bovine	UK	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
Zaf1	?	SA	4	1	2	2	2	1	2	1	−	+	F	−	INT	A		
97-v557	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A	Jan-97	
97-v562	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A	Jan-97	12
97-v572	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A	Jan-97	12
18156	Bovine	NL	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
3287 (ADRI 554)	Bovine	USA	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
3288 (ADRI 555)	Bovine	USA	4	1	2	2	2	1	2	1	−	+	V	+	V	A		
3295 (ADRI 1023)	Human	USA	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
44168	Bovine	NL	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
5.5.21	Bovine	NL	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
5.5.22	Bovine	NL	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
511	Bovine	HU	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
84-112	Bovine	USA	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
89-083	Bovine	USA	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
90-152	Bovine	USA	4	1	2	2	2	1	2	1	−	−	F	+	V	A		
95/258	Bovine	ARG	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
95-537 #2	Bovine	USA	4	1	2	2	2	1	2	1	−	+	V	+	V	A		
97/608	Bovine	ARG	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
99-257	Human	USA	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
BT 377/03	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
BT 74/00	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
LMG 6570 (LMD93,45)	Bovine	BE	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
NCTC 10354 (ATCC 19438)	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A		13
SA46	Bovine	ARG	4	1	2	2	2	1	2	1	−	−	F	+	V	A		
v311	Bovine	UK	4	1	2	2	2	1	2	1	−	−	V	+	V	A		
248,1	?	SA	5	1	1	1	2	1	1	3	+	+	F	−	F	B		
3051	Bovine	USA	5	1	1	1	2	1	1	3	+	+	F	−	F	B		
378/5	Bovine	BE	5	1	1	1	2	1	1	3	+	+	F	−	F	B	June-99	2
6877	Bovine	USA	5	1	1	1	2	1	1	3	+	+	V	−	F	B		
84-87	Human	USA	5	1	1	1	2	1	1	3	+	+	F	−	F	B		
87-580	Bovine	USA	5	1	1	1	2	1	1	3	+	+	V	−	F	B		
87-72	Bovine	USA	5	1	1	1	2	1	1	3	+	+	V	−	F	A/B		

Continued on following page

TABLE 1—*Continued*

Strain	Source	Country[a]	ST	Allele no.							Growth in glycine	H_2S production	Result by PCR of:		Result of AFLP analysis	*sap* type	Outbreak (mo-yr)	PFGE type
				aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*			Hum et al. (15)	Van Bergen et al. (36)				
97-742-1	Bovine	USA	5	1	1	1	2	1	1	3	+	+	V	−	F	B		4
DC 20C	Bovine	UK	5	1	1	1	2	1	1	3	+	+	F	−	F	B		
F8135	Human	GER	5	1	1	1	2	1	1	3	+	+	F	−	F	B		
Ru-11670-1	Bovine	NL	6	1	2	2	2	1	2	4	+	+	F	−	F	A	Dec-99	8
Ru-9516-18	Bovine	NL	6	1	2	2	2	1	2	4	+	+	F	−	F	A	Dec-99	8
0111-23289	Human	NL	6	1	2	2	2	1	2	4	+	+	V	−	F	A		
3754	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
4078	Human	BE	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
4079	Human	BE	6	1	2	2	2	1	2	4	+	+	V	−	F	A		
5.5.47	Ovine	NL	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
5515 (ATCC 33247)	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
5520 (ATCC 33561)	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
80-109	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
82-40	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
83-94	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
84-32 (23D)	Bovine	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
84-86	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
84-92	Bovine	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
88-060	Equine	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
88-231 T4	Bovine	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
94-130	Equine	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
99-256	Bovine	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
C036271	Human	UK	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
D223	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
D5605	Human	USA	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
E266	Human	BE	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
F128	Human	BE	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
Sz-1074	Ovine	NL	6	1	2	2	2	1	2	4	+	+	F	−	F	A		6
Tanaka	Human	JAP	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
Toda	Human	JAP	6	1	2	2	2	1	2	4	+	+	F	−	F	A		
BT 34/99	Bovine	UK	7	1	2	8	2	1	2	1	−	+	F	−	INT	A		
74	Ovine	TR	8	1	2	2	2	2	1	2	+	+	F	−	F	A		
97-365-1	Bovine	USA	9	1	2	2	2	1	3	4	+	+	F	−	F	A		5
L249093	Human	ESP	10	1	2	4	1	3	4	3	+	+	F	−	F	B		
5518 (ATCC 33293)	Human	USA	11	1	5	2	2	1	1	1	+	+	F	−	F	A		
Sa Cfv	?	SA	12	1	2	9	2	1	2	1	−	+	F	−	INT	A		
3290 (ADRI 802)	Ovine	USA	13	1	2	5	3	1	2	1	+	+	F	−	F	B		
3286 (small colony)	Ovine	USA	14	2	2	2	3	1	2	1	+	+	F	−	F	B		

[a] Abbreviations and symbols: NL, The Netherlands; UK, United Kingdom; TR, Turkey; USA, United States; BE, Belgium; SA, South Africa; F, France; ARG, Argentina; HU, Hungary; GER, Germany; JAP, Japan; ESP, Spain; ?, unknown; +, positive reaction; −, negative reaction; V, typed as *Campylobacter fetus* subsp. *venerealis*; F, typed as *Campylobacter fetus* subsp. *fetus*; INT, typed as *Campylobacter fetus* subsp *venerealis* bv. intermedius.

TABLE 2. Oligonucleotide primers used for *Campylobacter fetus* MLST

Locus	Sequence (5′ to 3′)	
	Forward primer	Reverse primer
aspA	CCT ATG ACT TTA GGT CAA GAG	TGT AGC TAG AGT ACG GCA AG
glnA	GAT GGT AGT TCT ATA GAC GC	CTT CCG TTA TCT CCA TAA AGC
gltA	CGA TAT AGC GTG GCT AGC TG	AGC GTG AGT AGA TCC TAC G
glyA	GAT AAA ATA CTT GGT ATG GAT C	CCC TCT GTT TAT TAA GAC TTC
pgm	AGA GTT GTT TTG GAC GTT GC	GTA GCT CAT CAA GAG GTC TC
tkt	GAG ATA GAT TGG TAT TTA GCG G	GTG ACT ACC TTC TAA ATC TCC
uncA	AAG AGT ACG GTG CTA TGG AC	CTC TCA TCA AGA TCG CTT GC

level of genetic diversity within this *Campylobacter* species; (ii) elucidate the *C. fetus* population structure and compare it to the population structures of other *Campylobacter* species; (iii) investigate any correlation between ST and cell surface antigen(s), represented by the *sap* type; and (iv) evaluate the utility of MLST for subspecies differentiation and epidemiological typing compared with those of AFLP analysis and PFGE.

All of the 140 isolates examined were typeable by MLST. The number of alleles per locus ranged from two to four, and the number of variable sites per locus ranged from one to five (Table 3). A total of 14 different STs were identified, some of which shared up to six or seven identical loci. The majority of isolates (91%) were assigned to one of the following STs: ST-2, ST-3, ST-4, ST-5, or ST-6 (Table 1). In this isolate collection, ST-1 was associated with an epidemiologically related group of isolates ($n = 5$), while the remaining STs (ST-7 to ST-14) each occurred only once.

The level of nucleotide sequence variation within the *C. fetus* MLST loci was assessed by concatenating the seven sequences to create a continuous 3,312-nucleotide length. There were 22 variable sites in the 14 STs (0.66%) (Table 4). The five most common STs had 12 variable sites and were apparently distributed along the sequence. Three of these mutations (25%) caused a change in the amino acid sequence.

C. fetus is genetically homogeneous with an apparently clonal population structure. In contrast, the five other *Campylobacter* species for which MLST data are available (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*) (4, 5, 21) show much greater diversity. This was illustrated by comparing data for the three loci used in all six *Campylobacter* MLST schemes described to date: *glnA*, *glyA*, and *tkt* (data for *glnA* are shown in Fig. 1). The variation detected in *C. fetus* is equivalent to a homogeneous *C. jejuni* clonal complex and is much less than that in many clonal complexes of this species (5).

The extent of association between the *C. fetus* STs (defined by housekeeping genes) and cell surface antigens was assessed by examining the relationship between the ST and the *sap* type (*sapA* or *sapB*). A clear correlation was observed (Table 1; Fig. 2). *SapA* was confined to isolates of ST-2, -4, -6, -7, -8, -9, -11, and -12, whereas *sap*B was associated with isolates of ST-1, -3, -5, -10, -13, and -14. Therefore, the association of this cell surface antigen with the ST demonstrated congruence.

***C. fetus* sequence type and subspecies.** The subspecies was defined by using the classical biochemical approach, PCR (15, 36), and AFLP analysis (39). An AFLP analysis with improved resolution and throughput (described here for the first time) allowed us to define a distinct cluster designated *C. fetus* subsp. *venerealis* bv. intermedius. This cluster contained the previously described *C. fetus* subsp. *venerealis* bv. intermedius strains 3292 and 3293 (31) and produced H_2S, and most isolates were negative for growth in glycine (Table 1). Three subspecies groups were therefore identified: *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *venerealis* bv. intermedius, and *C. fetus* subsp. *fetus*.

The value of MLST for subspecies determination was assessed. Certain STs did correlate with each of the three AFLP groups. A total of 55 of 57 of the *C. fetus* subsp. *venerealis* or *C. fetus* subsp. *venerealis* bv. intermedius isolates were ST-4 (Table 1; Fig. 2). The remaining 2 of the 57 isolates were ST-7 and ST-12, respectively; ST-12 differed by only one nucleotide from ST-4. The *C. fetus* subsp. *fetus* AFLP group showed a greater diversity of STs; but notably, ST-4, ST-7, and ST-12 were absent (Table 1; Fig. 2).

Investigation of *C. fetus* epidemiology by MLST. MLST data were examined for (i) the reliability of outbreak identification, (ii) any correlation of the ST with the isolation source or geographical region, and (iii) any correlation with and discrimination attained compared to the results of PFGE. Such data

TABLE 3. Allelic diversity within 140 *C. fetus* isolates

Enzyme	Gene	Fragment size (bp)	No. of alleles per locus	Variable sites	
				No. of nucleotides	No. of amino acids
Aspartase	*aspA*	477	2	1	1
Glutamine synthetase	*glnA*	477	3	2	0
Citrate synthase	*gltA*	402	4	5	3
Serine hydroxy methyl transferase	*glyA*	507	3	4	1
Phospho glucomutase	pgm	501	3	2	0
Transketolase	tkt	459	4	3	1
ATP synthase a subunit	*uncA*	489	4	5	1

TABLE 4. Variable sites identified within the 14 *C. fetus* STs within the concatenated 3,312-nucleotide sequences of the seven MLST loci

Subspecies and ST	Nucleotide substitution at the following locus and position:																					
	aspA	*glnA*		*gltA*					*glyA*				*pgm*		*tkt*			*uncA*				
	290	585	606	1027	1079	1311	1320	1344	1359	1665	1755	1827	2052	2256	2534	2700	2763	2850	2870	2940	2943	3273
C. fetus subsp. *fetus*																						
1	G	A	T	C	G	G	C	C	C	C	T	C	A	C	G	G	G	A	C	C	G	T
2		G					T	T	T	T	G								G			C
3		G					T	T			G	G					A					
5									T	T	G									T	A	
6		G					T	T	T	T	G						A	G				
8		G					T	T	T	T	G		G						G			C
9		G					T	T	T	T	G				A		A	G				
10		G						T						T		A				T	A	
11		G	C				T	T	T	T	G											
13		G			T		T	T			G	G					A					
14	T	G					T	T			G	G					A					
C. fetus subsp. *venerealis*																						
4		G					T	T	T	T	G						A					
7		G				A	T	T	T	T	G						A					
12		G		T			T	T	T	T	G						A					

were available for a subset of epidemiologically related and unrelated isolates ($n = 23$).

The collection of 140 isolates studied included isolates from seven known outbreaks (with two strains per outbreak) from different bovine AI stations (Table 1). All but one of the outbreaks were associated with STs frequently identified within this data set (ST-1 to ST-4 and ST-6). The remaining outbreak was associated with a single bovine ST-5 isolate from an additional AI station. Epidemiologically related strains shared identical STs and PFGE types (e.g., ST-1 and ST-2; Table 1). However, some epidemiologically unrelated strains had identical STs, but PFGE typing revealed discriminatory patterns (e.g., ST-3, ST-4, ST-5, and ST-6; Table 1), which indicated that PFGE has a greater discriminatory power.

The MLST data and the isolate data on host and geographical region were evaluated for potential associations. Bovine, ovine, and human isolates were the most numerous; but none of these hosts harbored a single ST. However, ST-1 ($n = 5$) and ST-4 ($n = 48$) were isolated almost exclusively from bovine hosts, the exceptions being two human isolates. Seven further ST-4 isolates (indicated by a question mark in Table 1) were considered likely (but unconfirmed) bovine isolates (M. Henton, personal communication). Therefore, with the possible exception of ST-4, which is the *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. intermedius ST and, therefore, by definition, cattle associated, no association between the ST and the host species was detected. The ST and the geographical region of isolation also lacked association, and all the common STs were found in Europe and North America.

DISCUSSION

An MLST scheme for *C. fetus* was developed, together with a database for the storage and exchange of MLST data and protocols (http://pubmlst.org/cfetus/). The scheme was evaluated by using 140 *C. fetus* isolates chosen for their diversity in terms of geography and host species. *C. fetus* lacked genetic diversity and had a high level of clonality compared to those of other *Campylobacter* species studied by MLST (4, 5, 21). This could be because mammalian *C. fetus* isolates evolved from a recent ancestor, or it may be due to a small population size and high-fidelity error-intolerant repair; or the genetic diversity of *C. fetus* may be limited by a lack of the natural competence found in other *Campylobacter* species.

C. fetus was originally divided into two subspecies by using host and niche preferences (38), but subsequent taxonomic studies led investigators to question this approach (14, 29). The difficulty of reliable subspecies differentiation reflects the fact that the two subspecies are extremely closely related genetically. For example, it proved impossible to differentiate subspecies by using the nucleotide sequences of two other housekeeping genes, *sapD* and *recA* (33), or the 16S rRNA gene (13, 25, 41). Since subspecies differentiation is of high statutory importance, the suitability of MLST for *C. fetus* subspecies differentiation was investigated. The *C. fetus* subsp. *venerealis* strains were all ST-4, although they were geographically and temporally distinct. The *C. fetus* subsp. *venerealis* bv. intermedius strains were also mostly ST-4, with two exceptions (one ST-7 strain and another ST-12 strain). Therefore, the MLST scheme did not allow differentiation between *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. intermedius strains. However, ST-4 was not detected among the *C. fetus* subsp. *fetus* isolates. Extension of the MLST scheme to other loci may reveal differences between *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. intermedius strains. The *C. fetus* subsp. *venerealis* "ST-4 clone" shows even less diversity within the housekeeping genes than the *C. fetus* subsp. *fetus* STs, supporting the theory of Véron and Chatelain, who proposed that *C. fetus* subsp. *venerealis* is a mutant of *C. fetus* subsp. *fetus* (38).

The data suggest that the MLST approach is reliable for subspecies differentiation since it was performed "blind" with-

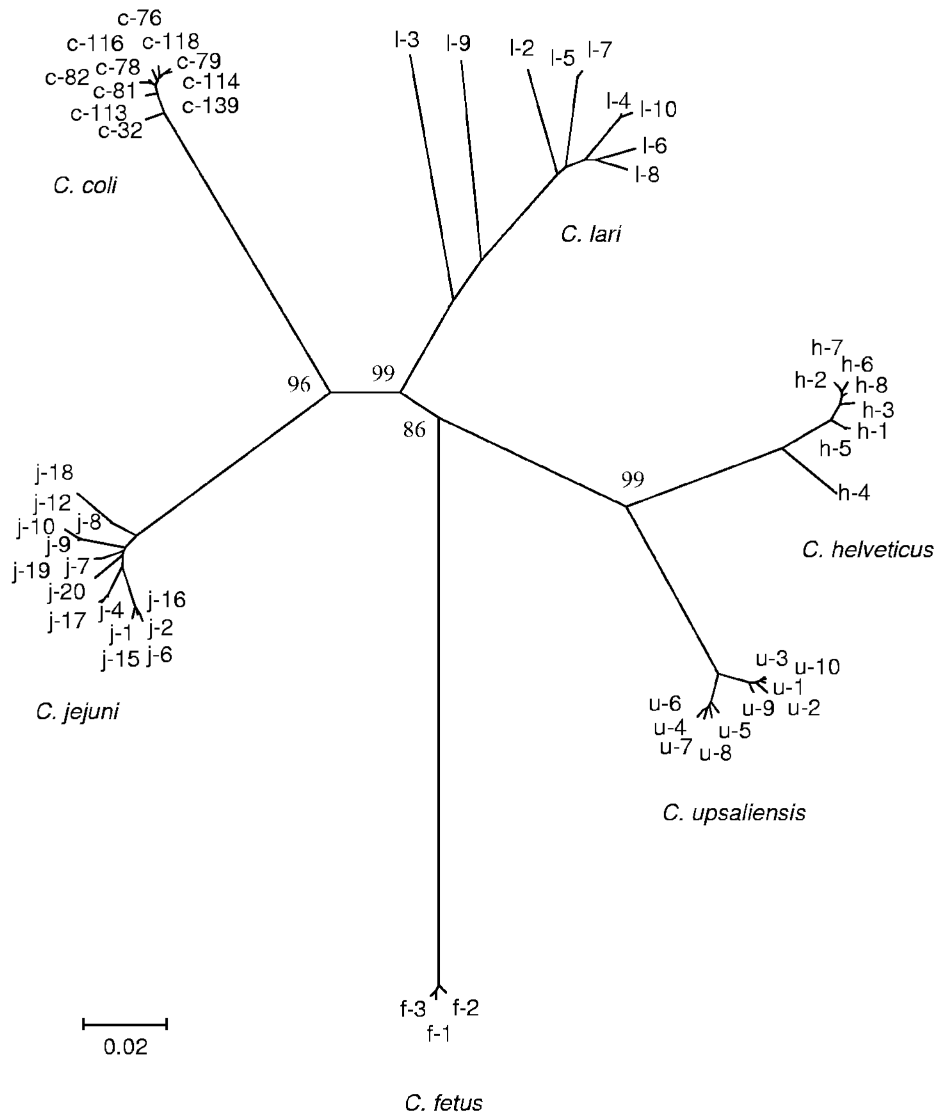

FIG. 1. Lack of genetic diversity in the *C. fetus glnA* locus compared to that in the *glnA* loci of other *Campylobacter* species. A radial neighbor-joining tree comparing the 477-nucleotide sequences at the *glnA* locus of *C. fetus* with those of five other *Campylobacter* species is shown. Alleles are numbered as in the MLST database at http://pubmlst.org/ and prefixed by a letter to indicate the species indicated. Bootstrap values are shown.

out prior knowledge of the subspecies assignments. Although *C. fetus* subsp. *venerealis* ST-4 differs from the closest *C. fetus* subsp. *fetus* ST (ST-6) by a single nucleotide (Fig. 2), the data generated by this highly reproducible sequencing technique confirm that MLST is an accurate method for the differentiation of the subspecies. Previous studies suggested that differences between *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* are plasmid encoded (25, 26), although more recent studies have questioned this proposal (42). This MLST scheme indicates that the differences between the two subspecies are not merely plasmid encoded but are also reflected in the allelic profiles (STs) of the housekeeping genes.

C. fetus isolates from mammals are divided into two *sap* types, *sapA* and *sapB*, on the basis of their SLPs. The MLST data showed a clear correlation among STs and *sap* types (Fig. 2), supporting the classification of *C. fetus* into two *sap* groups. This correlation suggests evolutionary stability and a lack of frequent recombination between the two groups, since in *C. jejuni* (which recombines frequently) a lack of correlation among STs-clonal complexes and cell surface antigens has been detected.

MLST is very useful for the global epidemiological typing of many bacterial species, and its use is expanding rapidly (18, 35). We demonstrated in the present study that epidemiologically related *C. fetus* strains had identical STs. However, as a result of the relatively low level of genetic diversity found in *C. fetus* housekeeping genes, some unrelated isolates had STs identical to those of some of the outbreak strains. Sequencing of additional, more variable loci has proven useful in investigations of outbreaks of *Neisseria meningitidis* infections and may increase the discriminatory power of the method (10). Whether this approach would also improve *C. fetus* discrimi-

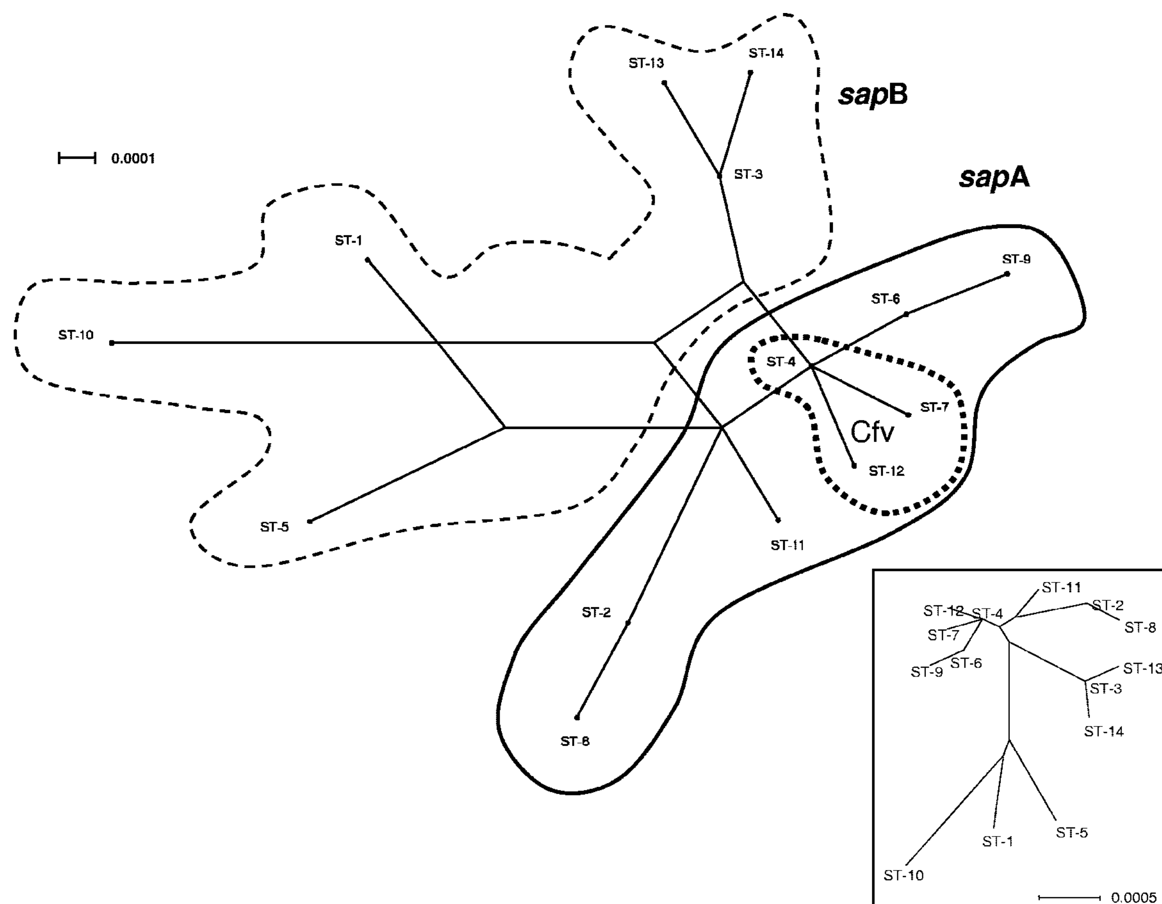

FIG. 2. Congruence of ST, *sap* type, and subspecies. Analysis of the concatenated MLST sequences was by split decomposition. The three STs identified in association with *C. fetus* subspecies *venerealis* (Cfv) are indicated by the dotted line; the remainder were associated with *C. fetus* subsp. *fetus*. The correlation among STs and *sap* types is marked; solid line, *sapA*; dashed line, *sapB*. (Box) A radial neighbor-joining tree constructed by using the same data is shown to indicate the corresponding tree-like phylogenies obtained by both methods. This supports the idea that *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* evolved recently, with little (if any) evidence of recombination; genetic changes have accumulated by the vertical transmission of point mutations, giving a clonal structure to the population.

nation requires further investigation. *C. fetus* has a global distribution; however, no geographically distinct ST cluster could be distinguished by MLST. The major STs were found in multiple host species; however, the *C. fetus* subsp. *venerealis* ST-4 was restricted to bovine hosts. It has been suggested that *C. fetus* subsp. *venerealis* is a host-restricted mutant clone of *C. fetus* subsp. *fetus* that is unable to infect multiple host species (38).

MLST was compared to biochemical and established *C. fetus* band-based typing methods, AFLP analysis, PFGE, and PCR. Each of these methods measures genotypic characteristics which may change independently; hence, the subtypes that they define may not always be congruent (30). However, MLST subspecies differentiation results showed a high agreement with AFLP, Cf C05 PCR, and H_2S results compared to the results of the PCR of Hum et al. (15) and the glycine test. In the present study, a strong correlation was observed between MLST and PFGE data, and both methods gave identical clusters of isolates from known *C. fetus* outbreaks. PFGE provided a higher level of discrimination than MLST. However, MLST measures variation that accumulates relatively slowly in the absence of selective pressure, and it may be more appropriate for studies of long-term *C. fetus* epidemiology and phylogenetics. PFGE indexes genome-wide variations and, despite the difficulties of interlaboratory comparisons, may be better suited for short-term investigations of *C. fetus* epidemiology, such as outbreak investigations, when such investigations are performed by a single laboratory. The level of discrimination of *C. fetus* isolates was higher by MLST than by the AFLP analysis method described, irrespective of the region of the AFLP pattern analyzed.

In summary, the observations that mammalian *C. fetus* isolates have a low level of genetic diversity and are genetically homogeneous compared to the homogeneities of other *Campylobacter* species suggest that *C. fetus* is young in evolutionary terms and may have arisen from a recent ancestor. In Fig. 1, the long branch length separating *C. fetus* from the other *Campylobacter* species suggests that its putative recent ancestor is missing from this tree. This is supported by the lack of any sequences characteristic of these five other *Campylobacter* species within the *C. fetus* housekeeping genes. The predominantly stepwise accumulation of point mutations in *C. fetus*

indicates clonal evolution, in which genetic change accumulates slowly only by the vertical transmission of point mutations. The genetic stability of *C. fetus* is further confirmed by the wide geographic distribution of identical STs and the observation of congruence between the *C. fetus* subspecies, *sap* type, and ST. The two *C. fetus* subspecies were extremely closely genetically related, but ST-4 was associated only with *C. fetus* subsp. *venerealis* and was therefore, by definition, a "bovine" subclone. MLST confirms that mammalian *C. fetus* is genetically stable and clonal, provides a useful additional tool for *C. fetus* subspecies differentiation and epidemiology, and compares favorably to other bacterial typing methods.

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