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Discovery of insertion element IS*Cfe1*: a new tool for *Campylobacter fetus* subspecies differentiation

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ABSTRACT

The species *Campylobacter fetus* is divided into the subspecies *C. fetus* subsp. *venerealis* (CFV) and *C. fetus* subsp. *fetus* (CFF). CFV is the causative agent of bovine genital campylobacteriosis, a highly contagious venereal disease that may lead to serious reproductive problems, including sterility and abortion. In contrast, CFF can be isolated from the gastrointestinal tract of a wide range of host species, is associated with abortion in sheep and cattle, and can also be isolated from local and systemic infections in humans. Despite differences in host and niche preferences, microbiological differentiation of the two subspecies of *C. fetus* is extremely difficult. This study describes the identification of a new insertion element, IS*Cfe1*, which is present exclusively in CFV strains, with highly conserved specific IS*Cfe1* insertion sites. The results are useful for identification and differentiation of the two *C. fetus* subspecies and will help in understanding the evolution and pathogenesis of *C. fetus*.

Keywords *Campylobacter fetus*, glycine tolerance, identification, insertion element IS*Cfe1*, subspecies differentation, taxonomy

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INTRODUCTION

The species *Campylobacter fetus* is divided into two subspecies, i.e., *C. fetus* subsp. *venerealis* (CFV) and *C. fetus* subsp. *fetus* (CFF), both of which are primary pathogens. CFV is characterised by a strong tropism for the genital tract of bovines, and is the causative agent of bovine genital campylobacteriosis, which is a contagious venereal disease that may lead to serious reproductive problems, such as sterility and abortion. Bovine genital campylobacteriosis is a notifiable disease of the Office International des Epizooties (OIE), and is considered to have significant socio-economic and public health implications, particularly with respect to the international trade of animals and animal products [1]. In contrast, CFF is isolated frequently from the gastrointestinal tract of a wide range of host species and is associated with abortion, most often in sheep and cattle [2]. CFF can also be isolated from local and systemic infections in humans, and is considered to be an underdiagnosed zooanthroponotic human pathogen because of its fastidious requirements for growth media and incubation atmosphere, and because selective media used routinely for isolation of *Campylobacter* spp. contain antibiotics, e.g., cephalothin and cefoperazone, to which *C. fetus* can be susceptible [3]. At present, the only accepted phenotypic method for differentiation of the two *C. fetus* subspecies relies on the fact that CFF can grow in the presence of glycine 1% w/v, while CFV cannot [2]. However, glycine tolerance in CFV can be mediated by phages, and differences in the level of glycine tolerance of CFF strains have been described [4].

Accurate detection and identification of *C. fetus* subspecies is essential for the implementation of efficient CFV control and eradication programmes, and for investigations into the public health burden of the two *C. fetus* subspecies. Several molecular methods, including pulsed-field gel electrophoresis fingerprinting and amplified fragment length polymorphism analysis, have been

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used for subspecies differentiation, but give contradictory results [5]. A PCR-based method to differentiate CFV from CFF has been proposed [6], based on the amplification of a random segment of genomic DNA, and this has been found useful by some investigators [7]. However, it has been reported that identification of CFV isolates originating from the UK could not be reliably confirmed using this PCR method [2]. Very few CFV-specific sequences, which would allow the creation of specific tests for *C. fetus* subspecies identification and differentiation, are contained in international public databases. The present study presents a novel subtyping method for *C. fetus*, based on the presence of a specific insertion sequence.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Details of the bacterial strains used in this study are shown in Tables 1 and 2. In addition to *Campylobacter* type and reference strains from bacterial culture collections, the study also included strains identified previously by the Swiss National Centre for Enteropathogenic Bacteria and the Institute of Veterinary Bacteriology using standard phenotyping methods [8]. Bacterial strains were cultured on tryptone soya agar containing sheep blood 5% v/v (Oxoid, Wesel, Germany) under microaerobic conditions for 2 days at 37°C. The identity of the *Campylobacter* strains used was confirmed by DNA–DNA hybridisation [9] and 16S rRNA and *rpoB* gene sequence analysis [10]. In addition, *C. fetus* subspecies identification was confirmed by investigating tolerance to glycine 1% w/v as described previously [7].

DNA extraction, PCR amplification and preparation of a CFV-specific DNA probe for Southern blotting

Campylobacter genomic DNA was extracted by the guanidium thiocyanate method as described previously [11]. All primers used in this study were synthesised by Mycrosynth (Balgach, Switzerland) and are listed in Table 3. PCR was performed in a DNA Fast Thermal Cycler 9800 (Applied Biosystems, Foster City, CA, USA) in 30-μL reaction mixtures containing 2.5 mM $MgCl_2$, 1 mM dNTPs, 3 μL of PCR buffer (ten-fold concentrate; Solis BioDyne, Tartu, Estonia), 2.5 U of FIREPol DNA polymerase (Solis BioDyne), 20 pmol of each primer and *c.* 50 ng of genomic DNA. PCRs comprised 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, the annealing temperature indicated in Table 3 for 30 s, and elongation at 72°C for the time indicated in Table 3, with a final elongation at 72°C for 10 min. Digoxigenin-11-dUTP (DIG)-labelled probes were produced by PCR using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Rotkreuz, Switzerland). A CFV-specific probe was prepared using primers IVB376-L and IVB376-R (Table 3) with genomic DNA from CFV strain NCTC 10354^T cloned into plasmid vector pBluescriptII SK(–) (Stratagene, La Jolla, CA, USA). In addition, all *C. fetus* strains used in this study were tested by the random segment-based PCR for *C. fetus* subspecies identification proposed by Hum *et al*. [6].

Southern blotting

Genomic DNA from different *C. fetus* strains was digested with *Bgl*II (which does not cut within the sequence used as a probe), electrophoresed on agarose 0.7% w/v gels and transferred to positively charged nylon membranes (Roche Diagnostics) using standard protocols [12]. The membranes were pre-incubated with 10 mL of hybridisation buffer (comprising 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM Na-citrate, pH 7), *N*-lauroylsarcosine 0.1% w/v, SDS 0.02% w/v and blocking reagent (Roche Diagnostics) 1% w/v) for each 100-cm^2 membrane at 68°C for 2 h, and were then hybridised overnight at 68°C with 5 mL of hybridisation buffer containing 1 μg of DIG-labelled probe/100-cm^2 membrane. The membranes were washed for 5 min at room temperature with 2 × SSC containing SDS 0.1% w/v, and twice for 15 min at 68°C with 0.2 × SSC containing SDS 0.1% w/v. The DIG-labelled probes were detected using phosphatase-labelled anti-DIG antibodies and CDP-*Star* (Roche Diagnostics) according to the manufacturer's instructions.

DNA sequencing and sequence analysis

DNA sequencing was performed with a DNA Sequenator AB 3100 and the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The sequences were completed by 'primer walking' using synthesised oligonuclotides, and were assembled using Sequencer v.4.6 software (GeneCodes, Ann Arbor, MI, USA). DNA sequence analysis was performed using Vector NTI Advance 10 software (Invitrogen, Basel, Switzerland), and comparisons to the NCBI sequence and protein databases were done by BLAST search analysis. The deduced amino-acid sequences were analysed using the PROSITE program [13].

Nucleotide sequence accession numbers

The CFV sequences have been deposited in the EMBL/GenBank database with the following accession nos: AM260752 for CFV insertion sequence IS*Cfe1* in strain NCTC 10354^T; AM86430 for the CFV *tnpA*, *tnpB* and *nahE* (partial) genes, truncated by insertion sequence IS*Cfe1*, in strain NCTC 10354^T; AM286431 for the CFV *tnpA*, *tnpB*, *metT* (partial) and *smtA* (partial) genes, truncated by insertion sequence IS*Cfe1*, in strain NCTC 10354^T; and AM286432 for the CFV *tnpB* (partial), *virB5* (partial) and *virB6* (partial) genes, truncated by insertion sequence IS*Cfe1*, in strain CCUG 34395.

RESULTS

Identification of *C. fetus* strains

All CFF strains were shown to have a glycine tolerance phenotype. Among the 26 CFV strains analysed, 25 failed to grow in the presence of glycine 1% w/v in the growth medium. Surprisingly, the CFV collection strain CCUG 34111 was glycine-tolerant (Table 1).

Table 1. *Campylobacter fetus* strains used in this study

Strain no.[a]	Clinical origin	Country of origin	Glycine tolerance[b]	PCR target sequences[c]					
				nahE	IS*Cfe1*	Hum PCR[d]	IS*Cfe1*– *nahE*	*metT*–IS*Cfe1*	VirB5–VirB5/VirB6 intergenic region
C. fetus subsp. *venerealis* strains									
NCTC 10354^T	Vaginal mucus	France	–	+	+	+	+	+	+
CCUG 538^T	Vaginal mucus	Unknown	–	+	+	+	+	+	+
CCUG 11287	Human blood	France	–	+	+	+	+	+	+
CCUG 7477	Bovine, abortion	France	–	+	+	+	+	+	–
CCUG 33900	Bovine, abortion	France	–	+	+	+	+	+	–
CCUG 33901	Unknown	France	–	+	+	+	+	+	+
CCUG 33936	Vaginal mucus	France	–	+	+	+	+	+	+
CCUG 33902	Bovine, preputial washing	Belgium	–	+	+	+	+	+	+
CCUG 33871	Unknown	Czech Republic	–	+	+	+	+	+	–
CCUG 33872	Unknown	Czech Republic	–	+	+	+	–	+	–
CCUG 24260	Bovine	Sweden	–	+	+	+	+	+	+
CCUG 34335	Unknown	Uruguay	–	+	+	+	+	–	+
CCUG 34111	Bovine, aborted foetus	Yugoslavia	+	+	–	–	–	–	–
CCUG 34394	Bovine	Argentina	–	+	+	+	+	+	+
CCUG 34395	Bovine	Argentina	–	+	+	+	–	–	+
CCUG 34396	Bovine	Argentina	–	+	+	+	–	–	+
NZ 4264/95	Bovine, aborted foetus	Australia	–	+	+	+	+	+	+
NZ 4266/95	Bovine, male	Australia	–	+	+	+	+	+	+
NZ 4267/95	Bovine, male	Australia	–	+	+	+	+	+	+
NZ 4268/95	Bovine, male	Australia	–	+	+	+	–	+	+
NZ 4269/95	Bovine, male	Australia	–	+	+	+	+	+	+
NZ 4270/95	Bovine, aborted foetus	Australia	–	+	+	+	–	–	+
NZ 4271/95	Bovine, placenta	Australia	–	+	+	+	+	+	+
NZ 4272/95	Bovine, aborted foetus	Australia	–	+	+	+	+	+	+
NZ 4273/95	Bovine, male	Australia	–	+	+	+	–	+	–
NZ 4274/95	Bovine, aborted foetus	Australia	–	+	+	+	–	+	+
C. fetus subsp. *fetus* strains									
ATCC 27374^T	Brain of sheep foetus	Unknown	+	+	–	–	–	–	–
ATCC 25936	Unknown	Unknown	+	+	–	–	–	–	–
CCUG 33671	Human peritoneal dialysis fluid	Sweden	+	+	–	–	–	–	–
CCUG 30605	Human blood	Sweden	+	+	–	–	–	–	–
CCUG 30043	Human blood	Sweden	+	+	–	–	–	–	–
CCUG 32676	Unknown	Canada	+	+	–	–	–	–	–
CCUG 32677	Unknown	Canada	+	+	–	–	–	–	–
K 57-5/91	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
K 58-1/91	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
K 34-1/91	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
K 49-2/91	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
K 54-4/91	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
NZ 305/94	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
NZ 754/94	Human	Switzerland	+	+	–	+	–	–	+
NZ 2231/94	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
NZ 2802/94	Human	Switzerland	+	+	–	–	–	–	–
NZ 3741/94	Human	Switzerland	+	+	–	–	–	–	–
NZ 2237/94	Bovine, faeces	Switzerland	+	+	–	–	–	–	–
NZ 10/95	Human	Switzerland	+	+	–	–	–	–	–
NZ 451/95	Human	Switzerland	+	+	–	–	–	–	–
NZ 1073/95	Human	Switzerland	+	+	–	–	–	–	–
N 53/03	Canine, faeces	Switzerland	+	+	–	–	–	–	–
N 971/03	Human	Switzerland	+	+	–	–	–	–	–
N 2054/03	Human	Switzerland	+	+	–	–	–	–	–
N 2150/03	Human	Switzerland	+	+	–	–	–	–	–
N 2765/03	Human	Switzerland	+	+	–	–	–	–	–
N 112/05	Human	Switzerland	+	+	–	–	–	–	–

[a]ATCC, American Type Culture Collection, Manassas, VA, USA; NCTC, National Collection of Type Cultures, London, UK; CCUG, Culture Collection of the University of Göteborg, Sweden; NZ, K, and N, Swiss National Centre for Enteropathogenic Bacteria, Bern, Switzerland.
[b]–, no bacterial growth; +, bacterial growth.
[c]–, negative result by PCR; +, positive result by PCR.
[d]Random segment-based PCR as described by Hum *et al.* [6].

Discovery of the insertion element IS*Cfe1*

After digestion of genomic DNA from CFF strain ATCC 27374^T and CFV strain NCTC 10354^T with restriction enzyme *Cfo*I, a fragment of *c.* 20 kb was found to be unique for CFV (not shown). The fragment was isolated by agarose gel extraction, and *Hin*dIII-generated fragments thereof were cloned into plasmid vector pBluescriptII SK(–). For identification of CFV-specific clones, colony blot hybridisation using DIG-labelled genomic DNA from CFF strain ATCC 27374^T was

Table 2. *Campylobacter* spp. (other than *Campylobacter fetus*) used in this study

Strain no.[a]	Species	Origin	Country	PCR target sequences[b]	
				nahE	IS*Cfe1*
IMD 1611/06	*C. jejuni*	Chicken	Switzerland	–	–
IMD 1612/06	*C. jejuni*	Chicken	Switzerland	–	–
IMD 1614/06	*C. jejuni*	Chicken	Switzerland	–	–
IMD 1636/06	*C. jejuni*	Bovine, faeces	Switzerland	–	–
IMD 1654/06	*C. jejuni*	Bovine, faeces	Switzerland	–	–
IMD 1394/06	*C. coli*	Bovine, faeces	Switzerland	–	–
IMD 1573/06	*C. coli*	Bovine, faeces	Switzerland	–	–
IMD 1578/06	*C. coli*	Bovine, faeces	Switzerland	–	–
IMD 1673/06	*C. coli*	Chicken	Switzerland	–	–
IMD 1700/06	*C. coli*	Bovine, faeces	Switzerland	–	–
IMD 1416/06	*C. hyointestinalis*	Bovine, faeces	Switzerland	–	–
IMD 1567/06	*C. hyointestinalis*	Bovine, faeces	Switzerland	–	–
IMD 1975/06	*C. hyointestinalis*	Bovine, faeces	Switzerland	–	–
K 35-3/91	*C. hyointestinalis*	Bovine, faeces	Switzerland	–	–
K 53-1/91	*C. hyointestinalis*	Bovine, faeces	Switzerland	–	–
CCUG 11290	*C. bubulus*	Bovine, sperm	Belgium	–	–
CCUG 886	*C. bubulus*	Bovine, sperm	UK	–	–
Ue 2808/05	*C. sputorum*	Bovine, preputial washing	Switzerland	–	–
Ue 2809/05	*C. sputorum*	Bovine, preputial washing	Switzerland	–	–
CCUG 12014	*C. sputorum* biovar *fecalis*	Ovine, faeces	Canada	–	–
ATCC 43157	*Arcobacter cryaerophilus*	Eye of aborted porcine fetus	Ireland	–	–
NZ 2303/99	*A. cryaerophilus*	Bovine	Switzerland	–	–
NZ 2978/99	*A. cryaerophilus*	Bovine	Switzerland	–	–

[a]ATCC, American Type Culture Collection, Manassas, VA, USA; NCTC, National Collection of Type Cultures, London, UK; CCUG, Culture Collection of the University of Göteborg, Sweden; NZ, K, and N, Swiss National Centre for Enteropathogenic Bacteria, Bern, Switzerland; IMD and Ue, Institute of Veterinary Bacteriology, ZOBA, Vetsuisse Faculty, Bern, Switzerland.
[b]–, negative result by PCR; +, positive result by PCR.

Table 3. Primers, targets and PCR parameters used in this study

Primers	Sequence (5′–3′)	Target sequences	Amplicon size (bp)	Annealing temperature (°C)	Elongation time
CFETSpp-L CFETSpp-R	GGTTATTTTTTATAACTGTAGGAATGCAGAT GATCGCTTAAATCTTGTACTTTTAGCTTTT	*nahE*	390	48	30 s
CVEN-L CVEN-R2	ATTAGTATTTGCAATATGTGAA AATTGATATTAAATTTGATTGA	IS*Cfe1*	233	48	30 s
AB-L CFETSpp-R	ACACACAAGGGATGATGA As above	IS*Cfe1*–*nahE*	836	48	1 min
CF-R CVEN-R2	AGAACACGTATCTACTGGATAT As above	*metT*–IS*Cfe1*	1064	48	1 min
pCA5-L AB-L	CTGGTATAAGAGATATAGTAAATTTCACGA As above	VirB5–IS*Cfe1*	1620	48	1 min
pCA5-L pCA5-R	As above TATCTCTTTGGTATGCTCTCCAGTTATTT	VirB5–VirB5/VirB6 intergenic region	420	50	30 s
IVB376-L IVB376-R	TAGCTTATGCAAGAGTTA TCCAAATCTTAATAATCT	CFV-specific sequence from pIVB376	230	48	30 s

performed. A plasmid clone containing a 687-bp CFV-specific fragment (which did not react with the CFF genomic probe) was identified and named pIVB376. Primers IVB376-L and IVB376-R (Table 3), targeting the pIVB376 CFV-specific fragment, were designed and used subsequently for the creation of a DIG-labelled CFV-specific probe. Simultaneously, a CFV *Bgl*II genomic library was constructed. The DIG-labelled probe was used for the identification of CFV *Bgl*II clones containing the CFV-specific DNA fragment. In this manner, a plasmid clone containing a DNA fragment of 2726 bp was found, sequenced and named pIVB412.

Characterisation of IS*Cfe1*

Analysis of the pIVB412 sequence identified two open reading frames, which were designated *tnpA* and *tnpB* because of their high similarity to other bacterial transposases (Fig. 1). The *tnpA* and *tnpB* genes were flanked by 12-bp inverted repeats and by 3-bp direct repeats (GGT). The inverted repeats, together with the *tnpA* and *tnpB* genes, constituted the structure of an insertion element, designated IS*Cfe1* by the International Depository ISfinder, Toulouse, France (www-is.biotoul.fr).

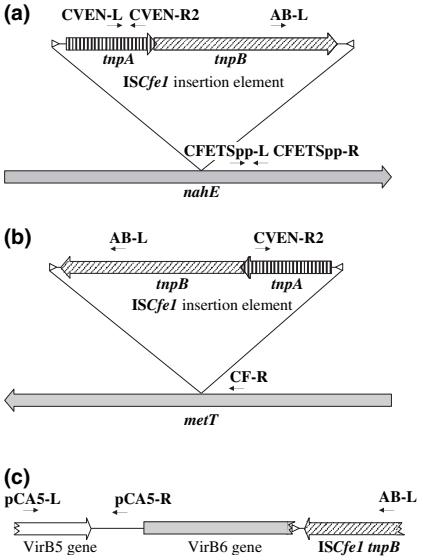

Fig. 1. Genetic map of the IS*Cfe1* insertion element and specific sites of insertion in: (a) the sodium/hydrogen exchanger protein gene *nahE*; (b) the putative methyltransferase protein gene *metT*; and (c) the putative VirB6 protein gene. Arrows dashed vertically and diagonally represent the genes of transposases A and B, respectively, of the insertion element IS*Cfe1*. Inverted repeats flanking IS*Cfe1* are indicated with small white triangles. Grey arrows indicate genes that are affected by the insertion element IS*Cfe1*. Small black arrows show the localisation of the primers used in the present study.

IS*Cfe1* insertion regions

The adjacent DNA region downstream of the IS*Cfe1* element in the pIVB412 clone was analysed. This DNA fragment showed 100% homology with the terminal part of the sodium/hydrogen exchanger protein encoded by the *nahE* gene of CFF (EMBL/GenBank accession number ZP_01073856). This indicates that the *nahE* gene is truncated by IS*Cfe1*, at least in the CFV type strain NCTC 10354^T. To investigate the presence of the *nahE* gene in strains of both *C. fetus* subspecies, primers CFETSpp-L and CFETSpp-R (Table 3, Fig. 1) were used. All CFF and CFV strains yielded a PCR product using these primers (Table 1). In contrast, the expected product was not observed when strains of other *Campylobacter* spp. were tested (Table 2).

PCRs using primer AB-L, targeting *tnpB* of IS*Cfe1*, and primer CFETSpp-R (Table 3, Fig. 1) were performed to determine whether the site of insertion of IS*Cfe1* in the putative *nahE* protein gene of the CFV strains was conserved. In 18 of the 26 CFV strains analysed, the IS*Cfe1*-specific insertion in the putative *nahE* gene was demonstrated (Table 1), and the DNA fragment containing the putative truncated *nahE* gene was identified in Southern blots (Fig. 2). The presence of an IS*Cfe1*-positive DNA fragment of *c.* 2.5 kb correlated with a positive PCR result using primer pair AB-L/CFETSpp-R. Strain CCUG 33871 gave a positive result when tested with the AB-L/CFETSpp-R PCR, probably because of differences in the localisation of *Bgl*II restriction sites in the genome, but the corresponding IS*Cfe1*-positive DNA fragment was not present in the Southern blot (Fig. 2). In contrast, the IS*Cfe1*-positive DNA fragment of *c.* 2.5 kb was observed in strain NZ 4270/95, although the same strain was negative when tested with the AB-L/CFETSpp-R PCR. This result was probably caused by mismatches in the DNA sequences of the PCR targets.

To identify other conserved IS*Cfe1* regions of insertion, as well as possible target genes that could prove useful for *C. fetus* subspecies differentiation, the DNA fragment corresponding to the IS*Cfe1*-positive band localised at *c.* 4.5 kb for CFV strain NCTC 10354^T (Fig. 2) was excised from the agarose gel, cloned and named plasmid pCA4. Additional IS*Cfe1*-positive DNA fragments yielding bands of <4.5 kb were found for strains CCUG 34395 and CCUG 34396. For this reason, the DNA corresponding to the IS*Cfe1*-positive DNA fragment of strain CCUG 34395 was cloned and sequenced. The plasmid containing this DNA fragment was named pCA5.

BLAST analysis of the flanking sequence downstream of the IS*Cfe1* element in plasmid pCA4 showed that IS*Cfe1* was inserted into the putative methyltransferase protein gene *metT*, which is present intact in CFF (EMBL/GenBank accession number NZ_AANR01000004.1) (Fig. 2). To confirm the specific site of insertion of the IS*Cfe1* element in the putative *metT* gene, a PCR was performed using primers CVEN-R2 and CFR (Table 3, Fig. 1). The IS*Cfe1*-specific insertion in the putative *metT* gene was demonstrated in 21 of the 26 CFV strains tested. Again, the presence of an IS*Cfe1*-positive DNA fragment of *c.* 4.5 kb correlated with positive PCR results using primers CVEN-R2/CFR (Table 1). CFV strains CCUG 34395 and CCUG 34396 yielded negative results when tested with primers CVEN-R2/CF-R (Table 1) and in Southern blot analysis (Fig. 2).

Two open reading frames flanking the IS*Cfe1* element in plasmid pCA5 were revealed.

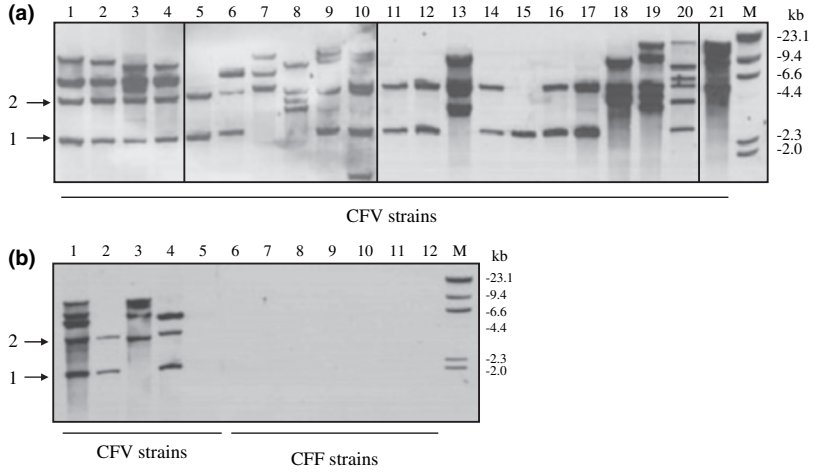

Fig. 2. Detection of the IS*Cfe1* element. Southern blots of genomic DNA from *Campylobacter fetus* subsp. *venerealis* (CFV) and *C. fetus* subsp. *fetus* (CFF) strains cut with *Bgl*II and probed with digoxigenin-11-dUTP (DIG)-labelled IS*Cfe1*. Strains analysed were as follows: (a) lane 1, type strain CCUG 538^T; lane 2, CCUG 11287; lane 3, CCUG 7477; lane 4, CCUG 33900; lane 5, CCUG 33936; lane 6, CCUG 33902; lane 7, CCUG 33871; lane 8, CCUG 33872; lane 9, CCUG 24260; lane 10, CCUG 34335; lane 11, NZ 4266/95; lane 12, NZ 4267/95; lane 13, NZ 4268/95; lane 14, NZ 4269/95; lane 15, NZ 4270/95; lane 16, NZ 4271/95; lane 17, NZ 4272/95; lane 18, NZ 4273/95; lane 19, NZ 4274/95; lane 20, CCUG 34394; and lane 21, CCUG 34396; (b) lane 1, type strain NCTC 10354^T; lane 2, NZ 4264/95; lane 3, CCUG 34395, lane 4, CCUG 33901; lane 5, CCUG 34111; lane 6, ATCC 25936; lane 7, CCUG 33671; lane 8, CCUG 32676; lane 9, CCUG 30605; lane 10, CCUG 30043; lane 11, K 34-1/91; and lane 12, K 49-2/91. M denotes molecular size standards. Arrows show highly conserved IS*Cfe1* element-positive bands. The presence of band 1 correlates with the IS*Cfe1* insertion into the sodium/hydrogen exchanger protein gene *nahE*. The presence of band 2 correlates with the IS*Cfe1* insertion into the putative methyltransferase protein gene *metT*. These two protein genes are present intact in CFF.

Following BLASTP analysis of the predicted protein sequences, a truncated TrbL/VirB6 putative conserved domain (NCBI pfam04610) was detected in the open reading frame flanking the IS*Cfe1* element. The predicted protein showed 27% identity and 50% similarity with the TrbL/VirB6 plasmid conjugal transfer protein of *Campylobacter lari* (EMBL/GenBank accession no. ZP_00369792). TrbL/VirB6 was preceded by another open reading frame with no putative conserved domains (Fig. 1). However, this predicted protein showed 25% identity and 50% similarity with the putative cmgb5 (VirB5) protein of *Campylobacter coli* (EMBL/GenBank accession no. YP_063430). The specific insertion of IS*Cfe1* in the TrbL/VirB6-like protein gene was verified by PCR analysis of strains CCUG 34395 and CCUG 34396 using primers pCA5-L and AB-L (Table 3, Fig. 1). Both strains were positive according to this PCR (results not shown).

Proteins VirB5 and VirB6 are components of a type IV secretion system (T4SS). T4SSs are present in various bacteria and are known to play a crucial role in bacterial pathogenesis. To investigate the occurrence of the T4SS in *C. fetus*, PCR was performed using primers pCA5-L and pCA5-R (Table 3, Fig. 1). Twenty of the 26 CFV strains tested were positive according to this PCR (Table 1), but among the CFF strains tested, only NZ 754/94 was positive (Table 1).

Distribution of IS*Cfe1* among CFV and CFF strains

All CFV strains contained at least one copy of the IS*Cfe1* element, as shown by Southern blot analysis, with the single exception of strain CCUG 34111 (Fig. 2). IS*Cfe1* was not present in any of the CFF strains tested (Fig. 2). There was no correlation between IS*Cfe1* hybridisation patterns and the geographical origin of the strains. To confirm the Southern hybridisation results by PCR, primers CVEN-L and CVEN-R2 targeting the IS*Cfe1* element were designed (Table 3, Fig. 1).

C. fetus subspecies differentiation based on IS*Cfe1*

The IS*Cfe1*-based PCR revealed that all CFV strains were positive for IS*Cfe1*, with the exception of strain CCUG 34111, while all CFF strains

tested were negative (Table 1). In addition, IS*Cfe1* was not detected in any other *Campylobacter* spp. tested (Table 2). When the results obtained with the CFU strains were compared with those obtained using the previously described PCR based on the random DNA segment [6], the results were in agreement, with the exception of results for strain CCUG 34111. When the CFF strains were tested, the random segment-based PCR was positive for strain NZ 754/94, as was also observed with the T4SS PCR (Table 1).

DISCUSSION

The IS*Cfe1* element was found to be highly conserved in CFV strains and, surprisingly, to be absent in CFF strains. A good correlation was found among the results of the glycine tolerance test, the IS*Cfe1* PCR, and the random segment-based PCR proposed by Hum *et al*. [6]. However, CFF strain NZ 754/94, which tolerates glycine 1% w/v in growth media and does not possess the IS*Cfe1* element, gave a positive result by the random segment-based PCR. These results indicate that the IS*Cfe1* PCR is a promising tool for *C. fetus* subspecies differentiation, but that the IS*Cfe1* PCR assay for *C. fetus* subspecies differentiation should be validated with larger numbers of strains of different origin, including those from the UK described by Willoughby *et al*. [2].

Based on identification of several IS*Cfe1* copies and a variable number of IS*Cfe1* elements among the CFV strains, it is tempting to speculate that IS*Cfe1* is able to spread in the genome by transposition. Moreover, the presence of the IS*Cfe1* element in all CFV strains strongly suggests that all CFV strains have a common ancestor. Interestingly, this insertion element is present in CFV strains independently of the geographical origin of the strain. No correlation was found between IS*Cfe1* hybridisation patterns or regions of insertion and the glycine intolerance phenotype of CFV. This indicates that the IS*Cfe1* element is probably not responsible for this intolerance.

It has been suggested that CFV is a host-restricted mutant clone of CFF that is unable to infect multiple host species [5]. In support of this hypothesis, it has been found that both subspecies of *C. fetus* are genetically extremely closely related [10] and, based on multilocus sequence typing, CFV has been postulated to be a 'bovine subclone' [5].

Bacterial insertion elements can be associated with gene rearrangements that may confer improved genetic fitness, which is important for the adaptive evolution of their host [14,15]. It can be hypothesised that IS*Cfe1* was acquired as a part of the evolutionary adaptation process in the bovine genital tract, and that this acquisition event took place before the worldwide spread of CFV strains. Fingerprinting of bacterial insertions is used frequently in epidemiological studies [16–18], but no evident correlation between IS*Cfe1* hybridisation patterns and the geographical origin of the strains was found in this study. However, a larger number of strains from different countries or regions should be included to investigate the use of IS*Cfe1* genome fingerprinting as a potential tool for epidemiological studies of *C. fetus*.

Based on Southern blot analysis and PCR results, two well-conserved IS*Cfe1* regions of insertion were cloned. One IS*Cfe1* element was inserted into a putative *nahE* gene and another into a putative *metT* gene. These two genes are apparently intact in CFF strains. The presence of IS*Cfe1* in the putative *nahE* and *metT* genes was shown in 69% and 80%, respectively, of the CFV strains tested. In addition, a PCR targeting only the putative *nahE* gene was shown to be a helpful tool for identification of both subspecies of *C. fetus.*

In two of the CFV strains analysed (strains CCUG 34395 and CCUG 34396), the putative VirB6 gene appeared to be truncated by a third IS*Cfe1* element. Further work is required to elucidate the effect of this truncation and, in particular, to elucidate the role of the T4SS in the pathogenesis of CFV infection. T4SSs are membrane-associated transporter complexes used by various bacteria to deliver effector molecules into a wide range of target cells. These molecules may affect, and alter, basic host cellular processes in different ways, resulting in the development of disease [19]. T4SSs have been described in large plasmids of various *Campylobacter* spp. [20,21], but this is the first report that indicates the presence of a T4SS in *C. fetus* subspecies. A putative T4SS was present in the majority (77%) of the CFV strains tested, but, surprisingly, in only one CFF strain.

Discrepancies were observed in the results for strain CCUG 34111. Although this strain has been characterised and stored as a strain of CFV in the Culture Collection of the University of Göteborg, Sweden, it was not possible to demonstrate the presence of IS*Cfe1*, either by Southern blot or by

PCR. At present, glycine tolerance is the only international phenotypic test accepted for *C. fetus* subspecies differentiation. This test is based on the ability of CFF strains to tolerate glycine 1% w/v. Moreover, *C. fetus* is considered to be a non-thermophilic *Campylobacter* sp. However, in a previous study, the majority of CFF strains showed growth at 42°C [7]. Interestingly, strain CCUG 34111 was able to grow at 42°C, had a glycine tolerance phenotype, and did not harbour IS*Cfe1*, making it similar to CFF.

In summary, differentiation of the subspecies of *C. fetus* is essential for the implementation of efficient CFV control and eradication programmes, and for investigating the public health burden of *C. fetus* subspecies. Despite the fact that *C. fetus* is an important pathogen for animals and humans, very few CFV sequences are deposited in international public databases, which hinders the creation of specific and reliable tests for *C. fetus* subspecies identification and differentiation. This, indirectly, reflects the limited knowledge of *C. fetus* biology and pathogenesis. The results of the present study provide some promising sequences that may be useful for *C. fetus* subspecies identification and differentiation, and for understanding the evolution and pathogenesis of *C. fetus*.

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