Comparative Genomics of Campylobacter fetus from Reptiles and Mammals Reveals Divergent Evolution in Host-Associated Lineages

Maarten J. Gilbert, William G. Miller, Emma Yee, Aldert L. Zomer, Linda van der Graaf-van Bloois, Collette Fitzgerald, Ken J. Forbes, Guillaume Méric, Samuel K. Sheppard, Jaap A. Wagenaar, and Birgitta Duim.

1Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands
2US Department of Agriculture, Produce Safety and Microbiology Research Unit, Agricultural Research Service, Albany, California
3WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, the Netherlands
4Biotechnology Core Facility Branch, Division of Scientific Resources, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta, Georgia
5School of Medicine and Dentistry, University of Aberdeen, United Kingdom
6College of Medicine, Institute of Life Science, Swansea University, United Kingdom
7MRC Cloud-Based Infrastructure for Microbial Bioinformatics (CLIMB) Centre, Swansea University, United Kingdom
8Department of Zoology, University of Oxford, United Kingdom
9Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands

*Corresponding author: E-mail: b.duim@uu.nl.

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Abstract

Campylobacter fetus currently comprises three recognized subspecies, which display distinct host association. Campylobacter fetus subsp. fetus and C. fetus subsp. venerealis are both associated with endothermic mammals, primarily ruminants, whereas C. fetus subsp. testudinum is primarily associated with ectothermic reptiles. Both C. fetus subsp. testudinum and C. fetus subsp. fetus have been associated with severe infections, often with a systemic component, in immunocompromised humans. To study the genetic factors associated with the distinct host dichotomy in C. fetus, whole-genome sequencing and comparison of mammal- and reptile-associated C. fetus was performed. The genomes of C. fetus subsp. testudinum isolated from either reptiles or humans were compared to elucidate the genetic factors associated with pathogenicity in humans. Genomic comparisons showed conservation of gene content and organization among C. fetus subspecies, but a clear distinction between mammal- and reptile-associated C. fetus was observed. Several genomic regions appeared to be subspecies specific, including a putative tricarballylate catabolism pathway, exclusively present in C. fetus subsp. testudinum strains. Within C. fetus subsp. testudinum isolated from either reptiles or humans were compared with elucidate the genetic factors associated with pathogenicity in humans. Genomic comparisons showed conservation of gene content and organization among C. fetus subspecies, but a clear distinction between mammal- and reptile-associated C. fetus was observed. Several genomic regions appeared to be subspecies specific, including a putative tricarballylate catabolism pathway, exclusively present in C. fetus subsp. testudinum strains. Within C. fetus subsp. testudinum, sapA, sapB, and sapAB type strains were observed. The recombinant locus iamABC (mlaFED) was exclusively associated with invasive C. fetus subsp. testudinum strains isolated from humans. A phylogenetic reconstruction was consistent with divergent evolution in host-associated strains and the existence of a barrier to lateral gene transfer between mammal- and reptile-associated C. fetus. Overall, this study shows that reptile-associated C. fetus subsp. testudinum is genetically divergent from mammal-associated C. fetus subspecies.

Key words: campylobacter fetus, reptile, mammal, comparative genomics, recombination, evolution.

Introduction

Campylobacter fetus has been recognized as a significant veterinary pathogen. Until recently, two subspecies were described: Campylobacter fetus subsp. fetus (Cff) and Campylobacter fetus subsp. venerealis (Cfv). Both subspecies have been isolated from multiple vertebrate hosts, mainly mammals, but the primary reservoir is considered to be
ruminants. These subspecies display distinct host and niche preferences: Cff is often associated with the intestinal tract and aborted fetuses of ruminants, mainly sheep and cattle, whereas Cfv is almost exclusively associated with the genital tract of cattle (van Bergen et al. 2008). Next to the aforementioned subspecies, a genetically distinct variant of C. fetus has been isolated from reptiles, with a reported prevalence of 5.5–6.7%, and humans (Harvey and Greenwood 1985; Tu et al. 2004; Dingle et al. 2010 Patrick et al. 2013; Wang et al. 2013; Gilbert, Kik, et al. 2014). This reptile-associated C. fetus has been described as C. fetus subsp. testudinum (Cft) (Fitzgerald et al. 2014).

Human infections caused by Cft have been reported and a reptilian origin in these cases is suspected (Tu et al. 2004; Patrick et al. 2013). In contrast to C. jejuni, symptoms of C. fetus-associated gastrointestinal illness are rarely reported (Wagenaar et al. 2014). Although cases of human Cft infection can be considered rare and opportunistic, and occur mostly in immunocompromised people, the systemic component in the majority of infections makes it difficult to treat and these infections are often life-threatening.

DNA sequence-based typing, including multilocus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) analysis, have shown that reptile-associated Cft are genetically distinct from mammal-associated Cff and Cfv and that genetic diversity is higher in Cft (Dingle et al. 2010; Fitzgerald et al. 2014). This suggests that reptile-associated evolutionary divergence between mammal- and reptile-associated C. fetus (Tu et al. 2001, 2005; Dingle et al. 2010; Kienesberger et al. 2014). Furthermore, the diversity among Cft isolates from reptiles is higher than the diversity among Cft isolates from humans (Dingle et al. 2010; Fitzgerald et al. 2014), which suggests that a subset of genotypes is able to colonize and potentially infect humans or that humans are selectively exposed to a subset of the population.

The genetic factors underlying host differentiation of mammal- and reptile-associated C. fetus are poorly understood. Comparing the whole-genome sequences of mammal- and reptile-associated C. fetus strains can provide valuable insights into this distinct host association, as well as further insights into speciation, taxonomy, and pathogenicity. In this study, 20 genomes of reptile-associated Cft strains were compared with 39 genomes of mammal-associated Cff and Cfv. To determine features specific to C. fetus, genomes of the most closely related species Campylobacter hyointestinalis and Campylobacter iguaniorum were included in the analyses. A phylogenetic reconstruction provided insights into the distinct host association of C. fetus. Furthermore, detailed genome analyses characterized genomic regions specific to C. fetus, and to Cft in particular, and revealed multiple species- and subspecies-specific sequence variations, including a distinct putative tricarballylate catabolism locus, and a genomic region associated with human invasive strains.

### Materials and Methods

#### Strains and Growth Conditions

A total of 61 strains were used for this study, including: 20 Cft strains of reptilian (n = 13) and human (n = 7) origin, 39 Cff and Cfv strains of bovine (n = 33), ovine (n = 1), human (n = 2), and unknown (n = 3) origin, one C. hyointestinalis strain of porcine origin, and one C. iguaniorum strain of reptilian origin. Characteristics of all strains used in this study are shown in table 1. Strains were grown on blood agar in a microaerobic atmosphere (83.3% N2, 7.1% CO2, 3.6% H2, and 6% O2) at 37°C for 48 h.

#### Genome Sequencing

In total, 61 genomes were included in this study (table 1). The whole-genome sequence data of 39 Cff and Cfv strains were from published studies (Stynen et al. 2011; Iraola et al. 2013; Barrero et al. 2014; Kienesberger et al. 2014; van der Graaf-van Bloois et al. 2014, 2016; Iraola et al. 2015). For 18 Cft strains, Illumina MiSeq reads were generated in this study; an average of 2.18 million Illumina MiSeq reads per strain were assembled using Newbler (v2.6), resulting in draft genomes containing, on average, 31 large contigs (>5,000 bp; average contig size = 61 kb) and 338 × coverage.

Whole-genome sequence data of Cft strains 03-427 and SP3 were obtained using a Roche 454 genome analyzer and was assembled into contigs using the Newbler assembler (v2.6). In this study, the genome of Cft strain SP3 was completed using methodology described previously for Cft strain 03-427 (Gilbert et al. 2013). In short, 221,254 Roche 454 reads were assembled into a single scaffold of 24 contigs using the Newbler assembler (v2.6). All Roche 454 base calls were validated using 1,852,600 Illumina MiSeq reads, providing a total coverage of 156 x. Sequences across the contig junctions and the S-layer (sap) locus were confirmed with Sanger sequencing. Assembly was confirmed using Pacific Biosystems long reads. PacBio RS reads were assembled into contigs using Quiver (Pacific Bioscience, Menlo Park, CA).

Campylobacter iguaniorum strain 1485E was sequenced as described previously (Gilbert, Miller, et al. 2014). The whole-genome sequence of C. hyointestinalis strain DSM 19053 was obtained from GenBank.

The completed genomes of Cft strains 03-427 and SP3, Cff strain 82-40, and Cfv strain 97/608 were used as reference genomes.

#### Genome Analysis

The genomes of Cft strains 03-427 and SP3 were annotated as described previously (Gilbert et al. 2013). Homopolymeric GC tracts were characterized using the high-depth MiSeq reads. CRISPR regions were identified using CRISPRFinder (Grissa et al. 2007). Genes were assigned a functional category using the RAST subsystem annotation approach as
Table 1
Features of the *Campylobacter* Strains Used in This Study

| Species | Strain | Host  | Source | Origin | MLST atpA sap wcdBK | Sequence Method | Level (contigs) | References | Accession Number |
|---------|--------|-------|--------|--------|----------------------|-----------------|-----------------|------------|-----------------|
| Cff     | 04/554 | Bovine| Fetus  | AR     | 5 3 B +              | 454, Illumina, PacBio | Complete        | van der Graaf-van Bloois et al. 2014 | CP0088808–CP008809 |
| Cff     | 82-40  | Human | Blood  | US     | 6 4 A                 | Complete        | Unpublished     | LMB000000000 |
| Cff     | 98v/445| Bovine| Prepuce| UK     | 3 1 B +              | Draft (192)     | Complete        | van der Graaf-van Bloois et al. 2014 | |
| Cff     | 80042  | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (25)      | van der Graaf-van Bloois et al. 2016 | ERR419595 |
| Cff     | 80047  | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (16)      | van der Graaf-van Bloois et al. 2016 | ERR419600 |
| Cff     | 80066  | Bovine| Feces  | UK     | 3 1 B +              | Illumina        | Draft (29)      | van der Graaf-van Bloois et al. 2016 | ERR419610 |
| Cff     | 80097  | Bovine| Feces  | UK     | 2 2 A                 | Illumina        | Draft (25)      | van der Graaf-van Bloois et al. 2016 | ERR419623 |
| Cff     | 80129  | Bovine| Feces  | UK     | 3 1 B +              | Illumina        | Draft (32)      | van der Graaf-van Bloois et al. 2016 | ERR419637 |
| Cff     | 80130  | Bovine| Feces  | UK     | 3 1 B +              | Illumina        | Draft (26)      | van der Graaf-van Bloois et al. 2016 | ERR419638 |
| Cff     | 80131  | Bovine| Feces  | UK     | 6 4 A                 | Illumina        | Draft (28)      | van der Graaf-van Bloois et al. 2016 | ERR419639 |
| Cff     | 80151  | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (19)      | van der Graaf-van Bloois et al. 2016 | ERR419648 |
| Cff     | 80152  | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (18)      | van der Graaf-van Bloois et al. 2016 | ERR419649 |
| Cff     | 80167  | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (19)      | van der Graaf-van Bloois et al. 2016 | ERR460866 |
| Cff     | 80168  | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (27)      | van der Graaf-van Bloois et al. 2016 | ERR460867 |
| Cff     | BT 1098| Ovine | Unknown| UK     | 2 2 A                 | 454             | Draft (44)      | van der Graaf-van Bloois et al. 2014 | LRAL00000000 |
| Cff     | H1-UY  | Human | Blood  | UY     | 4 1 A*                | Illumina        | Draft (34)      | Iraola et al. 2015 | JYCP000000000 |
| Cff     | 50693A | Bovine| Feces  | UK     | 5 3 B +              | Illumina        | Draft (20)      | van der Graaf-van Bloois et al. 2016 | ERR419284 |
| Cff     | 50478D | Bovine| Feces  | UK     | 3 1 B +              | Illumina        | Draft (24)      | van der Graaf-van Bloois et al. 2016 | ERR419653 |
| Cff     | 03-427 | Human | Blood  | US     | 15 6 A                | 454, Illumina, PacBio | Complete        | Gilbert et al. 2013 | CP006833 |

(continued)
| Species | Strain | Host | Source | Origin | MLST | atpA | sap | wcbK | Sequence Method | Level (contigs) | References | Accession Number |
|---------|--------|------|--------|--------|------|------|-----|------|----------------|----------------|------------|-----------------|
| Cft 1   | 11S02557-2 | Chelonian (Mauremys annamensis) | Feces | NL | 58 | 5 | A | – | Illumina | Draft (62) | This study | LFMJ00000000 |
| Cft 1   | 11S05168-1 | Snake (Python reticulatus) | Feces | NL | 59 | 5 | A | – | Illumina | Draft (109) | This study | LELD00000000 |
| Cft 12  | 500416-3 | Chelonian (Geochelone elegans) | Feces | NL | 60 | 5 | B | + | Illumina | Draft (52) | This study | LFJ00000000 |
| Cft 12  | 502225-3 | Lizard (Tiliqua rugosa) | Feces | NL | 61 | 5 | A | – | Illumina | Draft (54) | This study | LFLK00000000 |
| Cft 12  | 502263-3 | Chelonian (Pseudemys sp.) | Feces | NL | 62 | 5 | A | – | Illumina | Draft (116) | This study | LLLL00000000 |
| Cft 12  | 502842-30 | Chelonian (Aldabrachelys gigantea) | Feces | NL | 63 | 5 | B | + | Illumina | Draft (104) | This study | LFLM00000000 |
| Cft 12  | 502847-1 | Snake (Boa constrictor) | Feces | NL | 64 | 5 | AB | + | Illumina | Draft (148) | This study | LFLN00000000 |
| Cft 12  | 502835-1 | Snake (Orthriophis taeniurus) | Feces | NL | 65 | 5 | AB | + | Illumina | Draft (118) | This study | LFLO00000000 |
| Cft 12  | 504217-1 | Chelonian (Cuora mouhoti) | Feces | NL | 66 | 5 | B | + | Illumina | Draft (147) | This study | LFLP00000000 |
| Cft 13  | 500388-15 | Chelonian (Chelonoidis denticulata) | Feces | NL | 67 | 5 | A | – | Illumina | Draft (223) | This study | LFLO00000000 |
| Cft 85  | 387 | Chelonian (Terrapene carolina) | Feces | US | 16 | 5 | AB | + | Illumina | Draft (81) | This study | LFXC00000000 |
| Cft CFT7-2 | CF78-2 | Lizard (Tiliqua nigrolutea) | Feces | UK | 26 | 5 | A | – | Illumina | Draft (59) | This study | LFXD00000000 |
| Cft D4335 | Human | Feces | US | 31 | 5 | A | – | Illumina | Draft (56) | This study | LFXE00000000 |
| Cft D6659 | Human | Pleural fluid Hematoma | US | 15 | 6 | A | – | Illumina | Draft (54) | This study | LFXR00000000 |
| Cft D6683 | Human | Blood | US | 15 | 6 | A | – | Illumina | Draft (74) | This study | LFXX00000000 |
| Cft D6690 | Human | Feces | US | 15 | 6 | A | – | Illumina | Draft (137) | This study | LMBF00000000 |
| Cft D6783 | Human | Bile | US | 15 | 6 | A | – | Illumina | Draft (65) | This study | LMBE00000000 |
| Cft D6856 | Human | Feces | UK | 27 | 5 | A | – | 454, Illumina, PacBio | Complete | This study | CP010953 |
| Cv 4S  | 02/298 | Bovine | Fetus | AR | 4 | 1 | A | – | 454 | Draft (233) | van der Graaf-van Bloois et al. 2014 | LRVK00000000 |
| Cv 4S  | 03/293 | Bovine | Unknown | AR | 4 | 1 | A | – | 454, Illumina, PacBio | Complete | van der Graaf-van Bloois et al. 2014 | CP0006999–CP0007002 |
| Species | Strain | Host | Source | Origin | MLST | sap | wcbK | Sequence Method | Level (contigs) | References | Accession Number |
|---------|--------|------|--------|--------|------|-----|------|----------------|----------------|------------|-----------------|
| Cfv     | 03/596 | Bovine | Fetus  | AR     | 4    | 1   | A    | Draft          | (103)          | van der Graaf-van Bloois et al. 2014 | LRAM00000000 |
| Cfv     | 642-21 | Bovine | Uterus | AU     | 4    | 1   | A*   | Illumina       | (103)          | Barrero et al. 2014               | AJSG00000000 |
| Cfv     | 84-112 | Bovine | Genital secretion | US | 4    | 1   | A    | Complete       | (126)          | Kienesberger et al. 2014           | HG004426-HG004427 |
| Cfv     | 92/203 | Bovine | Placenta | AR | 4    | 1   | A    | Draft          | (133)          | van der Graaf-van Bloois et al. 2014 | LRVL00000000 |
| Cfv     | 97/532 | Bovine | Vagina | AR     | 4    | 1   | A    | Draft          | (125)          | van der Graaf-van Bloois et al. 2014 | LRER00000000 |
| Cfv     | 97/608 | Bovine | Placenta | AR | 4    | 1   | A    | 454, Illumina, PacBio | Complete | van der Graaf-van Bloois et al. 2014 | CP0008810–CP0008812 |
| Cfv     | 9825   | Bovine | Fetus  | AR     | 4    | 1   | A    | Draft          | (100)          | van der Graaf-van Bloois et al. 2014 | LRVL00000000 |
| Cfv     | 99541  | Bovine | Prepuce | AR | 4    | 1   | A*   | Illumina       | (126)          | Iraola et al. 2013                 | ASTK00000000 |
| Cfv     | ADRI 513 | Unknown | Unknown | AU | 4    | 1   | A    | Draft          | (103)          | van der Graaf-van Bloois et al. 2014 | LRFA00000000 |
| Cfv     | ADRI 1362 | Bovine | Unknown | AR | 4    | 1   | A    | 454           | Draft (98)     | van der Graaf-van Bloois et al. 2014 | LRER00000000 |
| Cfv     | B6     | Bovine | Vagina | AU     | 4    | 1   | A*   | Illumina       | (81)           | Barrero et al. 2014                | AJMC00000000 |
| Cfv     | B10    | Bovine | Unknown | US | 4    | 1   | A    | Draft          | (133)          | van der Graaf-van Bloois et al. 2014 | LRET00000000 |
| Cfv     | CCUG 33872 | Unknown | Abortion | FR | 4    | 1   | A    | Draft          | (101)          | van der Graaf-van Bloois et al. 2014 | LRLE00000000 |
| Cfv     | CCUG 33900 | Bovine | Abortion | FR | 4    | 1   | A    | Draft          | (53)           | van der Graaf-van Bloois et al. 2014 | LRKV00000000 |
| Cfv     | LMG 6570 | Bovine | Unknown | BE | 4    | 1   | A    | Draft          | (63)           | van der Graaf-van Bloois et al. 2014 | LREW00000000 |
| Cfv     | NCTC 10354 | Bovine | Vagina | UK     | 4    | 1   | A    | Draft          | (45)           | Stynen et al. 2011                | AFGH00000000 |
| Cfv     | WBT 011/09 | Unknown | Unknown | UK | 4    | 1   | A    | Draft          | (77)           | van der Graaf-van Bloois et al. 2014 | LMBI00000000 |
| Cfv     | Zaf 3  | Bovine | Fetus  | SA     | 4    | 1   | A    | Draft          | (59)           | van der Graaf-van Bloois et al. 2014 | LRZ00000000 |
| Cfv     | Zaf 65 | Bovine | Unknown | SA | 4    | 1   | A    | Draft          | (90)           | van der Graaf-van Bloois et al. 2014 | LRZY00000000 |
| Chy     | DSM 19053 | Porcine | Intestine | US | r/α  | r/α  | r/α  | IonTorrent    | Draft (26)     | Unpublished                       | JHQ00000000 |
| Gg      | 1485E  | Lizard (Pogona vitticeps) | Feces | NL | r/α  | r/α  | r/α  | Illumina, PacBio | Complete | Gilbert, Miller, et al. 2014 | CP009043–CP009044 |

**NOTE.**—Cig, C. iguaniuroid; Chy, C. hyointestinalis; AR, Argentina; AU, Australia; BE, Belgium; CZ, Czech Republic; FR, France; NL, Netherlands; SA, South Africa; UK, United Kingdom; US, United States; UY, Uruguay. MLST, multilocus sequence typing (sequence type); atpA, ATP synthase alpha subunit (MLST allele number); sap, sap type (A, B, or AB); wcbK, GDP-mannose 4,6-dehydratase (+, present; -, absent); r/α, not applicable.

*Predicted sap type based on sequence type and the absence of wcbK.

Subspecies designation inconclusive; subspecies fetus or venerealis based on phenotype; subspecies venerealis based on genotype (van der Graaf-van Bloois et al. 2014).
were considered (Overbeek et al. 2014). In the completed genomes, by comparison with a close relative, genes which were truncated due to a premature stop codon, frame-shifted, fragmented or had a missing start codon were defined as pseudogenes.

A local BLASTP, including all strains listed in table 1, was performed, based on the predicted proteomes of all genomes, and the results were screened for C. fetus species- and subspecies-specific features. To visualize genomic regions specific for C. fetus and C. fetus subsp. testudinum in particular, the BLAST ring image generator (BRIG) (Alikhan et al. 2011) was used at default settings, based on BLASTN v2.2.26. For this, the contigs of all Cft strains, Cff strain 82-40, and Cfv strain 97/608 were selected and the complete genome of Cft strain 03-427 was used as a reference.

The sap locus contains a high number of repeated sequences and its assembly can prove difficult. The genome of Cft strain 03-427 was used as a reference.

Phylogeny and Recombination Analysis

Recombination analysis of C. fetus was performed using Gubbins (Croucher et al. 2014). Briefly, open reading frames were predicted and annotated using Prokka (Seemann 2014) and all versus all BLAST was performed for all predicted proteins of the genomes (table 1) at an E-value cutoff of 1E-6. To determine the orthologous relationships of all proteins, the BLAST output was parsed by Orthagogue (Ekseth et al. 2014). Proteins were considered for orthology clustering if the proteins had at least 50% identity and at least 50% overlap. To determine the orthologous groups (OGs), Markov clustering (MCL) was performed using MCL-edge (Enright et al. 2002). Genes encoding the proteins were aligned with each other within their respective OGs using MUSCLE (Edgar 2004). A super alignment was created by concatenating the aligned genes according to their position in Cft strain 03-427 if they were present in all isolates. Gaps were removed using Gblocks (Castresana 2000). Recombination events were detected in this super alignment using Gubbins (Croucher et al. 2014) with the default settings. Phylogenetic dendrograms were created using Fasttree (Price et al. 2009).

Results and Discussion

General Features of the C. fetus subsp. testudinum Genomes

The circular genome size of Cft strains 03-427 and SP3 is 1.78 and 1.82 Mb, respectively, which is within the known size range of Campylobacter (1.53–1.97 Mb) and similar to Cff strain 82-40 (1.77 Mb) (Miller 2008) (supplementary table S1, Supplementary Material online). The average G+C content of both strains is 33.1%. The Cft strain 03-427 genome is predicted to contain 1,695 putative protein-coding genes, 43 tRNA genes, and three rRNA operons. Cft strain SP3 is predicted to contain 1,767 putative protein-coding genes, 40 tRNA genes, and three rRNA operons. No obvious mobile elements or plasmids were identified in either strain. Cft strain 03-427 contained 29 variable homopolymeric GC tracts (≥8 bp; 34 total GC tracts) and strain SP3 contained 24 variable homopolymeric GC tracts (≥8 bp; 29 total GC tracts).

Genetic Features Specific to C. fetus subsp. testudinum

Several genetic features specifically present or absent from Cft were identified (supplementary table S2, Supplementary Material online). A total of 33 genes was present in all Cft strains, but absent from all Cff and Cfv strains (supplementary table S3, Supplementary Material online). The most prominent difference identified between the C. fetus subspecies was a putative tcurABC locus (CFT03427_0075-0078), present in all Cft strains, but absent from Cff and Cfv (fig. 1). Proteins encoded by the tcurABC locus have been shown to function in the catabolism of tricarballylate (a citrate analog) (Lewis et al. 2004). It has been shown that Salmonella enterica serovar Typhimurium strain LT2 can use tricarballylate as a carbon and energy source, which feeds directly into the citric acid cycle (Gutnick et al. 1969; Lewis et al. 2004). These results suggest that tricarballylate could potentially be used as a carbon and energy source by Cft. A BLASTP analysis revealed that this pathway was also present in Campylobacter coli, Campylobacter cuniculorum, C. hyointestinalis subsp. lawsonii, C. iguaniorum, and C. jejuni. In C. hyointestinalis subsp. lawsonii, tcuR was absent, whereas in both Cft and C. iguaniorum isolated from reptiles tcurABC was complete. Noteworthy, tcurABC appears present mainly in Campylobacter taxa associated with hindgut fermenting vertebrates. This suggests that the pathway is conserved in Campylobacter taxa inhabiting a potentially similar intestinal niche where tricarballylate is available as a carbon and energy source.

A total of 23 genes was present in all Cff and Cfv strains, but absent from all Cft strains (supplementary table S3, Supplementary Material online). Interestingly, an aspartate racemase-encoding gene (CFF8240_1412) was present in all of the Cff and Cfv strains, but absent from Cft strains. As aspartate racemase catalyzes the conversion of L-aspartate to D-aspartate, Cft is predicted to be unable to convert L-aspartate to D-aspartate. As this was a pseudogene due to a premature stop codon in Cff 04/554, but not in the other completed Cff and Cfv genomes, and this gene is located in the lipooligosaccharide (LOS) biosynthesis locus (CFF8240_1399-1414) bound by waa genes and containing multiple glycosyltransferases (Gilbert et al. 2008), it is likely related to LOS structure, and one might expect expression to vary depending on serotype in Cff and Cfv.
Genomic features

1. tricarballylate catabolism region trcABC
2. nitrate/sulfonate/bicarbonate ABC transporter region
3. putative helicase (AAA domain)
4. S-layer region
5. ABC transporter region iamABC
6. S-layer region
7. S-layer-associated glycosylation region
8. CRISPR/Cas region
9. type III restriction/modification system
10. divalent anion:Na+ symporter (DASS) family protein
11. cytochrome c region
12. tricarboxylic acid transport system trcABC
13. type III restriction/modification system
14. transcriptional regulator (Crg family)
15. autotransporter domain protein
16. plasmid stabilization system protein/recombinase region
17. putative iron-regulated membrane protein
18. S-layer-associated glycosylation region
19. transcriptional regulator (Xre family)
20. endonuclease/methyltransferase region
21. ABC transporter region
22. O-linked glycosylation region
23. Nif sensor containing MCP-domain signal transduction protein
24. CRISPR/Cas system-associated RAMP superfamily proteins
25. bifunctional UGP-sugar hydrolase
26. carboxymuconolactone decarboxylase family protein region

Locus tag

CFT03427_0075-0078
CFT03427_0162-0166
CFT03427_0334-0335
CFT03427_0480-0482
CFT03427_0485-0494
CFT03427_0495-0499
CFT03427_0647-0663
CFT03427_0781-0784
CFT03427_0823-0824
CFT03427_0856-0859
CFT03427_0896-0898
CFT03427_0897-0898
CFT03427_0957-0958
CFT03427_0959-0958
CFT03427_1019-1020
CFT03427_1115
CFT03427_1197-1205
CFT03427_1277
CFT03427_1277
CFT03427_1352-1354
CFT03427_1352-1354
CFT03427_1387-1389
CFT03427_1508-1512
CFT03427_1523-1524
CFT03427_1543-1548
CFT03427_1625-1627
CFT03427_1628-1633
CFT03427_1697
CFT03427_1734-1739

**FIG. 1.**—BRIG plot of C. fetus. BLASTN-based genomic comparison of all Cft strains, including Cft strain 03-427 (reference genome), Cff strain 82-40, and Cfv strain 97/608. Cft strains isolated from humans are shown in blue; Cft strains isolated from reptiles are shown in green, with predicted Cft sapA strains shown in dark green and predicted Cft sapB and sapAB strains shown in light green; Cff is shown in yellow; and Cfv is shown in red. Characteristic genomic features of C. fetus and Cft in particular have been highlighted. Features containing only hypothetical proteins are not indicated.
Genetic Features Specific to C. fetus

Several genetic features were identified in C. fetus, but were absent from the related species C. hyointestinalis and C. iguaniorum (supplementary table S2, Supplementary Material online). In total, 65 genes were present in all C. fetus strains, but absent from both C. hyointestinalis and C. iguaniorum strains (supplementary table S3, Supplementary Material online).

Genes encoding succinyl-CoA synthetase alpha and beta subunits (suuDC; CFT03427_0903-0904), proline dehydrogenase (napA; CFT03427_1218) and sodium/proline symporter (putP; CFT03427_1219), hemin ABC transporter system (chuaABCD; CFT03427_1703-1706), and serine protease (CFT03427_1733) were present in all C. fetus strains examined, but absent from its closest relatives.

In addition to catalase (CFT03427_1038), another catalase-like protein encoding region (CFT03427_1708) was present in all C. fetus strains examined. In Cff 82-40 and Cfv 97/608 however, this was a probable pseudogene. In parallel with catalase, which catalyzes the decomposition of hydrogen peroxide to water and oxygen and is present in many Campylobacter species, this catalase-like protein might be involved in protection against oxidative damage by reactive oxygen species.

The L-fucose permease-encoding gene fucP and the surrounding coding region (CFT03427_1042-1047) was present in all C. fetus strains examined, but was absent from its closest relatives C. iguaniorum and C. hyointestinalis. Low homology orthologs (57–65% identity) were identified in C. coli and C. jejuni by an online BLASTP search against the nonredundant database. In some C. jejuni strains, fucP and the surrounding coding region (cjo480c-cjo490) are implicated in the uptake of the sugar L-fucose, which is released from the host’s mucin glycoproteins and has been shown to be important in colonizing hosts (Muraoka and Zhang 2011; Stahl et al. 2011). Noteworthy, fucP and the surrounding coding region were present in C. coli showing introgression by C. jejuni genes, suggesting that the presence of this region provides C. coli an advantage in the gastrointestinal tract (Sheppard et al. 2013). Fermentation of carbohydrates is considered uncommon in Campylobacter, which might be related to the absence of 6-phosphofructokinase needed for glycolysis and key enzymes in the Entner-Doudoroff pathway (Parkhill et al. 2000; Kelly 2008). However, the presence of fucP and the surrounding coding region in C. fetus predicts that, besides amino acids and organic acids, L-fucose can be metabolized by C. fetus. In parallel with C. jejuni, this region might be involved in colonization of the host’s gastrointestinal tract.

Prophage and Foreign DNA Defense Mechanisms

In Cft strain SP3, a 35,178 bp putative prophage was present between a leucyl tRNA and the cas genes of the CRISPR/Cas system. No known toxins or virulence factors were identified within this putative prophage. In Cft strain 03-427, no prophages were identified, although a region encoding hypothetical proteins with unknown function was identified in the same CRISPR/Cas region, between the leucyl tRNA and the cas genes. In the complete genomes of the Cff and Cfv strains examined, this location contained phage-like elements in Cff strains 82-40 and 04/554, but not in Cfv.

CRISPRs were identified in the complete genomes of Cft strains 03-427 and SP3. Six genes coding for the CRISPR-associated proteins Cas1-6 (CFT03427_0656-0663) were conserved in all 20 Cft strains examined. However, these cas genes were identified in only 20.5% (8/39) of the Cff and Cfv genomes (supplementary table S2, Supplementary Material online). Noteworthy, all Cfv strains were lacking the cas genes, while eight Cff strains, including strains 82-40, B0131, and JYCP01, which are most closely related to Cfv, did contain these genes. Although the cas genes were absent from Cfv strain 97/608, this strain did contain CRISPRs. The presence of CRISPRs suggests that cas genes may have been present, but have been lost in Cfv strain 97/608. Notably, the cas genes identified in C. fetus were not homologous with cas genes found in other Campylobacter species and showed highest homology with Sulfurospirillum and Sulfurovum species.

Interestingly, four additional CRISPR/Cas system-associated RAMP superfamily protein coding loci (CFT03427_1628-CFT03427_1633) were conserved in 93.2% (55/59) of the C. fetus strains (supplementary table S2, Supplementary Material online). These genes are largely confined to C. fetus, although orthologs were identified with low homology in C. concisus and C. rectus. The exact function of these proteins is unknown. No CRISPRs were identified surrounding these genes, suggesting that this cluster of genes is unlike currently known CRISPR/Cas systems.

Virulence Determinants and Surface Structures

Most known and predicted virulence determinants, such as cadF, ciaB, and cytolethal distending toxins cdhABC, identified in C. fetus previously (Ali et al. 2012), were also identified in Cft. Two adjacent genes encoding a hemagglutinin/haemolysin-related protein (CFT03427_0734) and a haemolysin secretion/activation protein (CFT03427_0735) were identified in all C. fetus strains examined, but not in C. hyointestinalis and C. iguaniorum. Additionally, a patatin-like phospholipase (CFT03427_1717) was exclusively found in C. fetus. In Pseudomonas aeruginosa, a patatin-like protein has been linked to the development of lung injury, sepsis, and bacterial dissemination in animal models and human infections (Banerji and Flieger 2004). Type IV secretion system related gene clusters, such as virB genes, were commonly present in Cfv and in Cff strain 98/445 (van der Graaf-van Bloois et al. 2016), but were absent from Cft (supplementary table S2, Supplementary Material online).
One of the most prominent and distinguishing surface structures in *C. fetus* is the S-layer, which is associated with resistance to complement-mediated killing and is considered to be an important virulence factor (Blaser et al. 2008). The *C. fetus* S-layer is encoded by the *sap* locus, which contains the conserved *sapCDEF* locus and multiple copies of either *sapA* or *sapB*, and occasional *sapAB* recombinants. The completed genomes of Cft strains 03-427 and SP3 were predicted to contain eight *sapA* copies. Of all Cft strains examined, 70% (14/20) were *sapA* type, 15% (3/20) were *sapB* type, and 15% (3/20) were *sapAB* type (table 1). Remarkably, the *sapCDEF* locus was not observed in Cft strain D6683, Cff strains B0047 and S0478D, and CfV strains 642-21, CCUG 33900, and ADRI 513 (supplementary table S2, Supplementary Material online). Considering the high variability of this region and the draft nature of the genomes, the sequence reads of these particular strains were searched for parts of these genes and were found absent in the reads as well. As these genes are essential in formation of the S-layer (Blaser et al. 2008), these particular strains may be unable to form an S-layer. The inability to form an S-layer, associated with an 8–9 kb deletion in the sap locus, has been shown before in spontaneous *C. fetus* mutants (Oworkin et al. 1995).

Adjacent to the sap region, 14 Cft strains contained a conserved glycosylation region associated with *sapA* (CFT03427_0495-0499), whereas the conserved glycosylation region characteristic for *sapB* (CFF04554_0484-0487), including GDP-D-mannose dehydratase-encoding *wcbK* (Kienesberger et al. 2014), was identified in six Cft strains isolated from reptiles, which were *sapB* and *sapAB* type strains (table 1). These latter strains were also missing another glycosylation region (CFT03427_1352-1354), which was also absent from all mammal-associated *sapB* type *C. fetus* strains, but was present in all *sapA* type *C. fetus* strains examined, suggesting it is associated with glycosylation in *sapA* type *C. fetus* (supplementary figs. S1 and S2, Supplementary Material online). The two different conserved glycosylation regions adjacent to the sap locus are likely involved in LPS-biosynthesis and Sap binding, as has been shown for *wcbK* in *sapB* type *C. fetus* (Kienesberger et al. 2014).

In seven of the Cft strains isolated from reptiles, including all predicted *sapB* and *sapAB* type strains, the region containing *flaAB* and the motility accessory factor *maf* (CFT03427_1587-1590) was highly divergent from all other *C. fetus* strains examined, and *flaAB* and *maf* showed highest homology with the related species *C. hyointestinalis* and *C. iguaniurum* (Gilbert, Miller, et al. 2014), suggesting recombination between Cft and a strain closely related to these taxa, which was supported by recombination analysis. Recombination of *flaAB*, possibly due to selective pressure of the host immune response, has been shown in *C. jejuni* (Wassenaar et al. 1995).

Remarkably, a large region (~49 kb) adjacent to *flaAB*, encoding many O-linked glycosylation-related proteins (CFT03427_1543-1586), was conserved in all Cff/Cfv and most Cft strains examined, but was absent from the seven *flaAB* recombinant Cft strains (fig. 1). Within this region, genes encoding multiple glycosyltransferases and two asparagine synthases were identified. Of the 29 variable homopolymeric GC tracts identified within the Cft strain 03-427 genome, 12 hypervariable GC tracts (41%) were located within this glycosylation region. In *C. jejuni*, O-linked glycosylation is associated with flagellar assembly and function (Guerry 2007), and genes in the pseudaminic acid biosynthetic pathway (*pseB* and *pseH*) are found in and adjacent to this region. The strong association with *flaAB* in the nonrecombinant strains suggests that this region is involved in the O-linked glycosylation of flagellin. Like in the *flaAB* recombinant Cft strains, this region was missing in *C. iguaniurum* (Gilbert, Miller, et al. 2014). No other comparable glycosylation region was found conserved in all recombinant *flaAB* Cft strains, suggesting differential glycosylation of flagellin in these strains.

Interestingly, a region encoding ABC-transporters *iamABC* (also annotated as *mlaFED*; CFT03427_0480-0484), and showing higher than expected amino acid homology (99–100%) between all Cff and CfV strains and human clinical Cft strains, but not reptilian Cft strains (93–97%), was observed. Recombination analysis indicated a recombination event between Cff and Cft isolated from humans. A maximum-likelihood phylogenetic dendrogram based on *iamA* (*mlaF*) illustrated this recombination event (fig. 2). This recombination was identified in 71.4% (5/7) of the Cft strains isolated from humans, but was not identified in strains isolated from reptiles. Although from different geographical origins (Dingle et al. 2010; Patrick et al. 2013), all strains with this potential recombination were closely related and belonged to the same sequence type (ST15). Notably, all these Cft strains were *atpA* (uncA) MLST allele type 6, whereas all other Cft strains known are MLST allele type 5 (Dingle et al. 2010; Wang et al. 2013; Gilbert, Kik, et al. 2014), which corresponds to one point mutation. A closer inspection of the clinical background of the Cft strains isolated from humans revealed that all of the recombinant strains were isolated from bile, blood, a hematoma, or pleural fluid, whereas the nonrecombinant strains were isolated from stool samples (table 1), suggesting that the recombinant strains may be invasive. Orthologs of *iamABC* are found across the *Campylobacter* genus. Notably, in all *C. fetus* strains examined, the *iamABC* region is conserved amidst the highly recombining sap region. The ABC-transporter encoded by *iamA* is considered a virulence factor associated with invasion in *C. jejuni* (Carvalho et al. 2001). Most *C. fetus* infections in humans are systemic or have a systemic component and the ratio of systemic infection to diarrheal illnesses for *C. fetus* is much higher than for *C. jejuni*, indicating a propensity for invasive disease compared with *C. jejuni* (Blaser et al. 2008; Patrick et al. 2013; Wagenaar et al. 2014). The mechanisms of invasion in *C. fetus* are poorly understood; however, considering both mammal-
and reptile-associated *C. fetus* with recombinant *iamABC* show invasion, the *iamABC* region may have a similar function in invasive *C. fetus* strains.

**Campylobacter fetus** Phylogeny

*Campylobacter fetus* is generally considered a genetically coherently species with low genetic diversity compared with some other *Campylobacter* species (van Bergen et al. 2005). A phylogenetic reconstruction accounting for the effects of homologous recombination was performed for *C. fetus* and the most closely related species, based on a 781,293 nt gapless alignment (fig. 3). Mammal- and reptile-associated *C. fetus* formed two clearly separated clades. This is in line with earlier observations that mammal- and reptile-associated *C. fetus* form two distinct clades with an average amino acid identity of 95–96% and supports the description of *C. fetus* subsp. *testudinum* as a novel subspecies (Fitzgerald et al. 2014). The core genome phylogeny confirmed that genetic
diversity was higher among Cft than among Cff and Cfv. A shorter branch length suggests that Cft is more closely related to the last common ancestor. Genetic diversity was higher in Cft isolated from reptiles, which may be related to a larger diversity among the sampled reptile population. All invasive Cft strains isolated from humans showed little genetic diversity among each other and formed a separate lineage together with Cft strain D6783, distinct from the Cft strains isolated from reptiles, confirming earlier observations based on MLST and AFLP (Dingle et al. 2010; Fitzgerald et al. 2014).

The number of single nucleotide polymorphisms (SNPs) detected inside recombinations was higher in Cft, whereas the number of SNPs outside recombinations was lower in Cft, compared with Cff and Cfv (table 2). Also the ratio of base substitutions predicted to have been imported through recombination to those occurring through point mutation (r/m) and the ratio of the number of recombination events to point mutations (rho/theta) were higher in Cft. This indicates that mutation through recombination is more important than mutation through point mutation in Cft, compared with Cff and Cfv, based on the strains in this study.

Predicted recombinant regions were subdivided into regions shared by two or more strains, indicated by red blocks, or unique regions, indicated by blue blocks (fig. 3). More shared recombination events were detected in Cff/Cfv than in Cft. In contrast, more unique recombination events were detected in Cft strains isolated from reptiles. However, the invasive Cft strains isolated from humans and Cft strain 1485E (DSM19053) 03427 D6783 D6659 D6683 D6856 12S0284230 12S028471 85387 12S022253 12S004163 12S042171 12S028551 12S022633 D4335 CF782 11S025572 13S0038815 11S051681 SP3 98v445 B0066 B0130 B0129 NC101 04554 NC147 B0167 B0168 B0047 B0151 B0152 B0042 B0097 BT1098 JYCP01 8240 B0131 64221 ADRI513 CCUG33872 Zaf3 97608 84112 B10 CCUG33900 LMG6570 NCTC10354 WBT01109 Zaf65 03293 97532 92203 03596 9825 99541 ADRI1362 02298 8700 84650 37200 21621 85390 71621 62631 25021 67601 10621 13621

**Fig. 3.**—Phylogenetic reconstruction of the *C. fetus* clade based on Gubbins. Recombination regions within the 781,293 nt gapless core genome alignment are indicated in red (similar recombination region in multiple strains) or blue (unique recombination region). Species and subspecies from top to bottom: *C. iguaniorum*, purple; *C. hyointestinalis*, dark blue; *C. fetus* subsp. *testudinum* (human strains), light blue; *C. fetus* subsp. *testudinum* (reptilian strains), dark green (sapA strains) or light green (sapB and sapAB strains); *C. fetus* subsp. *fetus* (based on genotype), yellow (sapB strains) or orange (sapA strains); *C. fetus* subsp. *venerealis* (based on genotype), red. For *C. fetus*, the sap type of the corresponding strains is indicated with A, B, or AB. To increase the intraspecies resolution for *C. fetus*, the branches of the dendrogram are truncated for *C. hyointestinalis* and *C. iguaniorum*. Recombination regions of interest have been highlighted.
Table 2
Summary of the Recombination Analysis for Cft and Cff and Cfv

|                              | Cft  | Cff and Cfv |
|------------------------------|------|-------------|
| Total SNPs                   | 25,562 | 62,228      |
| Number of SNPs inside recombinations | 7,970   | 3,103       |
| Number of SNPs outside recombinations | 17,592  | 59,125      |
| Number of recombination blocks | 107    | 56          |
| Bases in recombinations      | 497,384 | 820,273     |
| r/m                          | 0.453  | 0.052       |
| rhotheta                     | 0.006  | 0.001       |
| Genome length                | 781,293 | 781,293     |

Note.—The ratio of base substitutions predicted to have been imported through recombination to those occurring through point mutation is indicated by r/m. The ratio of the number of recombination events to point mutations is indicated by rhotheta.

D6783 showed no unique recombination events, which supports the close genetic relationship of these strains.

A clear association between phylogeny and sap type was observed in Cff and Cfv, with sapA and sapB type strains forming separate clusters (fig. 3). This confirms the previously shown correlation between MLST sequence types and sap types (van Bergen et al. 2005). However, in Cft no association between phylogeny and sap type was observed, which may be explained by the larger influence of recombination in Cft. The presence of sapAB recombinant Cft strains also suggests that the sap locus is less conserved in Cft. Reptile-associated Cft can be sapA, sapB, or sapAB, which is in contrast to previous studies (Tu et al. 2005; Dingle et al. 2010). The identification of these sap types in genetically diverse Cft strains shows that these sap types are widespread in Cft and are present in both mammal- and reptile-associated C. fetus. In contrast to previous studies (Tu et al. 2005; Kienesberger et al. 2014), this suggests that both sap types were likely present before the mammal- and reptile-associated C. fetus lineages diverged, although it cannot be excluded that sap types recombined between mammal and reptile-associated C. fetus at a later stage. The presence of sapAB chimeras confirms that recombination of these different sap types occurs. As such, the diversity of the sap locus, which encodes a surface antigen under diversifying selection imposed by the host immune response, may hamper evolutionary assumptions.

Despite the different, well-separated niches, and genome-wide genetic divergence, mammal- and reptile-associated C. fetus are similar in overall gene content and synteny. In this study, only a few recombination events between mammal- and reptile-associated C. fetus were observed, suggesting that recombination between mammal- and reptile-associated C. fetus can be considered rare and that effective barriers to recombination exist, likely due to the separate host reservoirs, although other factors, such as genetic divergence and other intrinsic factors inhibiting recombination cannot be excluded. This is consistent with allopatric speciation. In contrast, Cff and Cfv show a nearly identical core proteome (van der Graaf-van Bloois et al. 2014), and different niche preferences are potentially associated with laterally acquired elements in Cfv (Ali et al. 2012; Kienesberger et al. 2014). Indeed, within C. fetus, Cfv showed the largest (accessory) genomes. Based on the high genetic similarity between Cff and Cfv, and the lack of a clear boundary between these subspecies, the validity of these subspecies has been questioned (van Bergen et al. 2008; van der Graaf-van Bloois et al. 2014). Nevertheless, Cff and Cfv do show small but consistent genetic differences based on phylogenetic analyses, indicating genetic divergence of these two lineages, which could be explained by an ecological barrier in which Cff and Cfv do not or barely recombine despite a high degree of niche overlap, as has been demonstrated for C. jejuni (Sheppard et al. 2014). Alternatively, the low genetic diversity of C. fetus may be explained by a lack of natural competence, as observed in vitro (Tu et al. 2003; Kienesberger et al. 2007, 2014), or, conversely, by a high rate of homologous recombination within the different C. fetus lineages, maintaining lineage coherence. With the advent of whole-genome sequencing, recombination is considered more common than previously assumed and observed clonality does often not exclude recombination (Feil et al. 2001), as was identified in this study for C. fetus.

Conclusions

The genomes of C. fetus subsp. testudinum in this study show high conservation in gene content and synteny compared with other C. fetus subspecies. Although the similarity between reptile- and mammal-associated C. fetus is high, the genomes are clearly divergent in overall sequence identity and in gene content.

Most notable features shared by the majority of C. fetus strains in this study are the S-layer, a CRISPR/Cas system and CRISPR/Cas system-associated RAMP superfamily proteins unique for Campylobacter. Several genomic differences were observed between mammal- and reptile-associated C. fetus, of which the presence of a putative tricarballylate metabolism pathway in Cft is most notable. In contrast to earlier observations, C. fetus subsp. testudinum can contain sapA, sapB, or sapAB. In seven Cft strains isolated from reptiles the region containing flaAB and maf was highly divergent. These strains were lacking a large adjacent glycosylation region conserved in all other C. fetus strains.

Recombination between mammal- and reptile-associated C. fetus seems rare, indicating effective barriers to recombination between these two divergent lineages, likely due to the separate host reservoirs, consistent with allopatric speciation. Nevertheless, a recombination event of iamABC between mammal-associated C. fetus and Cff strains isolated from humans was observed, which is associated with invasion in humans.
The whole-genome sequences of reptile-associated C. fetus subsp. testudinum provide a better understanding of C. fetus as a species and genomic features associated with subspecies, host type, and virulence, and provide further insights in C. fetus biology and evolution.

Supplementary Material
Supplementary figures S1 and S2 and tables S1–S3 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org).

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