Comparative Genomics of \textit{Campylobacter iguaniorum} to Unravel Genetic Regions Associated with Reptilian Hosts

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\section*{Abstract}
\textit{Campylobacter iguaniorum} is most closely related to the species \textit{C. fetus}, \textit{C. hyointestinalis}, and \textit{C. lanienae}. Reptiles, chelonians and lizards in particular, appear to be a primary reservoir of this \textit{Campylobacter} species. Here we report the genome comparison of \textit{C. iguaniorum} strain 1485E, isolated from a bearded dragon (\textit{Pogona viticeps}), and strain 2463D, isolated from a green iguana (\textit{Iguana iguana}), with the genomes of closely related taxa, in particular with reptile-associated \textit{C. fetus} subsp. \textit{testudinum}. In contrast to \textit{C. fetus}, \textit{C. iguaniorum} is lacking an S-layer encoding region. Furthermore, a defined lipooligosaccharide biosynthesis locus, encoding multiple glycosyltransferases and bounded by \textit{waa} genes, is absent from \textit{C. iguaniorum}. Instead, multiple predicted glycosylation regions were identified in \textit{C. iguaniorum}. One of these regions is > 50 kb with deviant G + C content, suggesting acquisition via lateral transfer. These similar, but non-homologous glycosylation regions were located at the same position on the genome in both strains. Multiple genes encoding respiratory enzymes not identified to date within the \textit{C. fetus} clade were present. \textit{C. iguaniorum} shared highest homology with \textit{C. hyointestinalis} and \textit{C. fetus}. As in reptile-associated \textit{C. fetus} subsp. \textit{testudinum}, a putative tricarballylate catabolism locus was identified. However, despite colonizing a shared host, no recent recombination between both taxa was detected. This genomic study provides a better understanding of host adaptation, virulence, phylogeny, and evolution of \textit{C. iguaniorum} and related \textit{Campylobacter} taxa.

\textbf{Key words:} Campylobacter iguaniorum, reptile, comparative genomics, recombination, phylogeny, evolution.

\section*{Introduction}

The majority of the \textit{Campylobacter} species are associated with endothermic mammalian and avian hosts. Recently, a novel \textit{Campylobacter} species has been described, \textit{Campylobacter iguaniorum} (Cig), which is predominantly isolated from ectothermic reptilian hosts (Gilbert et al. 2015). Chelonians and lizards in particular appear to be a primary reservoir of this \textit{Campylobacter} species. Reported overall prevalence in reptiles based on culturing was 8.2%; 15.6% of the chelonians and 6.1% of the lizards, but none of the snakes examined, carried this \textit{Campylobacter} species (Gilbert et al. 2014a). Recently, Cig has also been isolated from a non-reptilian host (Miller et al. 2016a).

Within the genus \textit{Campylobacter}, this species forms a clearly separated phylogenetic clade, together with the closely related taxa \textit{C. fetus}, \textit{C. hyointestinalis}, and \textit{C. lanienae} (collectively called the \textit{C. fetus} clade). \textit{C. fetus} currently comprises three subspecies: mammal-associated \textit{C. fetus} subsp. \textit{fetus} (Cff) and \textit{C. fetus} subsp. \textit{venerealis} (Cfv), and reptile-associated \textit{C. fetus} subsp. \textit{testudinum} (Cft) (Fitzgerald et al. 2014). As with Cig, Cft has been shown to be associated primarily with reptiles, and both taxa are the most frequently isolated \textit{Campylobacter} in reptiles (Harvey and Greenwood 1985; Wang et al. 2013; Gilbert et al. 2014a). Also, \textit{C. hyointestinalis} has been infrequently isolated from reptiles (Gilbert et al. 2014a). Interestingly, \textit{Campylobacter} species commonly

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found in various avian and mammalian hosts, such as C. coli, C. jejuni, and C. lari, were not isolated from reptiles, despite culturing conditions suitable for these species. It was speculated that the host body temperature, which is on average lower and more fluctuating in reptiles than in mammals and birds, is associated with this remarkable species distribution (Gilbert et al. 2014a). Indeed, Cig, C. fetus, and to a lesser extent C. hyointestinalis, were found to grow at lower temperatures than most other Campylobacter species (Gilbert et al. 2015).

The pathogenicity of Cig in reptiles is unknown; isolates have been recovered both from reptiles with and without clinical signs of disease (Benejat et al. 2014; Gilbert et al. 2014a). In contrast to reptile-associated Cft, which has been shown to cause infection in humans (Tu et al. 2004; Patrick et al. 2013), no human Cig infections have been reported to date.

The first closed and annotated Cig genome has been described previously (Gilbert et al. 2014b). Genome analysis and comparison provide valuable insights into host adaptation, virulence, phylogeny, and evolution of this reptile-associated Campylobacter species. Comparison with reptile-associated Cft could identify factors specific for adaptation to their shared reptilian host. Here we report the complete whole genome sequences of two Cig strains isolated from reptiles, and compare them to those of the closely related taxa Campylobacter fetus, C. hyointestinalis, and C. larienae.

**Campylobacter iguaniorum** genome features and comparison

The genome size of strain 1485E is 1,684,608 bp with a 70,030 bp megaplasmid; strain 2364D is 1,809,624 bp with an estimated 54,764 bp megaplasmid. The general features of Cig strains 1485E and 2463D are summarized in supplementary table S1, Supplementary Material online.

Multiple genomic regions were specific for Cig, i.e. no orthologs were identified in all or most members of the C. fetus clade or the entire Campylobacter genus. In total, 59 genes were conserved in both Cig strains, which were absent from all other C. fetus clade members (supplementary table S2, Supplementary Material online).

Multiple genes involved in sulfur metabolism were identified, indicating that this is important in Cig biology. Indeed, phenotypic testing showed that Cig and C. hyointestinalis are one of the few Campylobacter species that produce H2S on sulfate containing TSI agar (Gilbert et al. 2015). Notably, the gene coding for L-lactate permease (lctP) was absent from Cig and C. hyointestinalis subsp. hyointestinalis, but was well conserved in the other C. fetus clade members and most other Campylobacter species. In C. jejuni, transport of L-lactate occurs primarily via LctP, although at least one other transport route also exists (Thomas et al. 2011). This predicts that Cig relies less on exogenous L-lactate as carbon or energy source than other Campylobacter species encoding lctP.

Several genes were found to be shared specifically by both reptile-associated taxa Cig and Cft (supplementary table S2, Supplementary Material online). As in reptile-associated Cft, a putative tricarballylate catabolism locus tcuRABC (CIG1485E_0479-0482; CIG2463D_0480-0483) was identified in both Cig strains. This locus is partially present in C. hyointestinalis subsp. lawsonii, but as tcuC is a truncated pseudogene, it is likely not functional. Tricarballylate could potentially be used as a carbon and energy source by Cig, as this locus has been shown to function in the catabolism of tricarballylate (a citrate analog) (Lewis et al. 2004). Tricarballylate is considered to cause grass tetany in ruminants, a disease characterized by acute magnesium deficiency. In the ruminant rumen, trans-aconitate present in grass is rapidly reduced to tricarballylate, a toxic end product of ruminal fermentation (Russell 1985). Neither the ruminant nor the normal rumen flora can catabolize tricarballylate efficiently. However, it has been shown that *Salmonella enterica* serovar Typhimurium strain LT2 can use tricarballylate as a carbon and energy source; the end product of tricarballylate metabolism is cis-aconitate, which then enters into the citric acid cycle (Gutnick et al. 1969; Lewis et al. 2004). Conservation of the tcuRABC locus in the reptile-associated taxa suggests that tricarballylate is ubiquitous in the niche inhabited by Cig and Cft, i.e., the mucosa of the reptilian intestines. As the tcuRABC locus is present in all highly prevalent reptile-associated Campylobacter taxa, this locus could confer an advantage in colonization of the reptilian host.

In contrast to most Campylobacter species, both Cig strains lack a defined lipooligosaccharide (LOS) region, bounded by waa genes and containing multiple glycosyltransferases (supplementary table S2, Supplementary Material online). The lack of waaDEF, which are highly conserved in Campylobacter and Arcobacter, is especially remarkable. Instead, multiple separate predicted glycosylation regions were identified (fig. 1). A large glycosylation region (>50 kb) was present in both strains. Although syntopic in both strains, large parts of this region were not homologous and, in combination with the deviant G+C content (29.0–30.0%), suggests acquisition via lateral transfer. However, this region was highly homologous in strain 2463D and C. hyointestinalis subsp. hyointestinalis. In strains 1485E and 2463D, respectively 20.6% (7/34) and 22.0% (9/41) of the GC tracts were identified within this glycosylation region.

As in other Campylobacter species many of the hypervariable GC tracts reside in surface structure-related genes showing phase variation. However, in strains 1485E and 2463D, respectively 23.5% (8/34) and 20.0% (8/41) of the GC tracts were located within autotransporter domain-containing genes, whose role in Cig biology remains to be determined. In total, seven autotransporter domain-containing genes were found conserved in both strains and showed no or low
**FIG. 1.**—BRIG plots of *C. iguaniorum* and most closely related *Campylobacter* species. BLASTN-based genomic comparison of *C. iguaniorum* strains 1485E and 2463D, *C. fetus* subsp. testudinum (Cft) strains 03-427 and SP3, *C. fetus* subsp. fetus (Cff) strain 82-40, *C. fetus* subsp. venerealis (Cfv) strain 97/608, *C. hyointestinalis* subsp. *hyointestinalis* (Chh) strain LMG 9260, *C. hyointestinalis* subsp. *lawsonii* (Chl) strain CCUG 27631, and *C. laniensae* (Clan) strain NCTC 13004. Characteristic features of *C. iguaniorum* have been highlighted. Reference genome is *C. iguaniorum* strain 1485E (A); reference genome is *C. iguaniorum* strain 2463D (B).
homology with other Campylobacter species. Noteworthy, 89% (8/9) of the autotransporter domain-containing genes in both strains contained a GC tract.

The cas genes present in Cig were homologous with those in C. hyointestinalis subsp. hyointestinalis, but not with those in C. fetus, indicating that at least two different CRISP/Cas systems are present within the Campylobacter fetus clade (fig. 1).

One of the characteristics shared by Cig, C. fetus, and most Arcobacter species is the ability to grow at lower temperatures (<25 °C) than most other Campylobacter species (On et al. 1996; Gilbert et al. 2015). Also, these species have been shown to occur together in reptilian hosts, which often show a broad body temperature range and on average lower body temperatures compared with most endothermic mammals and birds (Gilbert et al. 2014a). As in C. fetus, but in contrast to C. jejuni in which nusE and nusO are lacking (Kelly 2008), all NADH:quinone oxidoreductase complex I subunits (NuoA-N) are present in Cig, suggesting that NADH is an important electron donor in this species. Interestingly, NuoA-N present in Cig and all other C. fetus clade members showed higher homology to species from the Arcobacter genus (>72%), than to other species from the Campylobacter genus (>55%). The high homology observed in the NADH:quinone oxidoreductase complex I subunits might be related to low temperature adaptation. Indeed, in other organisms NADH:quinone oxidoreductase complex I is considered the most thermolabile protein complex of oxidative phosphorylation (Downs and Heckathorn 1998). Within the Campylobacter genus, the ability to exclusively proliferate at lower temperatures (18–37 °C) is unique for Cig. This feature may have helped Cig colonize hosts which have a low and variable body temperature.

**Campylobacter iguaniorum** phylogeny and diversity

A phylogenomic reconstruction accounting for the effects of homologous recombination was performed, based on a 1,042,737 nucleotide gapless alignment of the whole genomes of Cig and most closely related species C. fetus, C. hyointestinalis, and C. lanieneae (supplementary fig. S1, Supplementary Material online). Cig was clearly distinct from the other Campylobacter species. The first split occurred between C. lanieneae and the other species. Excluding the more distantly related C. lanieneae, the branch lengths indicated that Cig is less related to the last common ancestor than the other species. Interestingly, Cig, C. fetus, and C. hyointestinalis branch of the last common ancestor at the same point, suggesting that these species started diverging at the same time.

In order to examine the genomic relatedness in further detail, the average nucleotide identity (ANI) was determined for the whole genomes of both Cig strains and strains of the most closely related taxa C. fetus, C. hyointestinalis, and C. lanieneae (supplementary table S3, Supplementary Material online). ANI values were highest for Cig and both C. hyointestinalis and C. fetus. Homology was higher between Cig and reptile-associated Cft than between Cig and mammal-associated C. fetus subspecies fetus and venerealis, indicating that both reptile-associated taxa Cig and Cft share genomic regions, which might be associated with adaptation to their shared reptilian hosts. Higher ANI values between Cig strain 2463D and Cft strain SP3, C. fetus subs. venerealis, and C. hyointestinalis subsp. hyointestinalis can be explained by shared laterally transferred genomic regions, such as prophages, and shared glycosylation regions (fig. 1). In addition to this, the genes shared by Cig and other members of the C. fetus clade (>50% identity) showed that most genes were shared between Cig and C. hyointestinalis subsp. hyointestinalis, followed by both Cft strains, suggesting that these taxa are most closely related (supplementary table S4, Supplementary Material online).

MLST analysis of 18 Cig strains showed two distinct lineages and a high intraspecies diversity without any identical sequence type (fig. 2). No clear association with host type was observed, as Cig isolates originating from lizards and chelonians were found in both lineages. Nevertheless, host association was observed at the species level to some degree, as Cig isolates originating from animals of the same species, but from different locations were mostly clustering together. This was most clear in iguana iguana, in which two Cig isolates, which were obtained from different animals in 2003 and 2012, clustered closely together. In contrast, two isolates obtained from the same animal (11S02571-1 and 11S02571-4) showed a large genetic distance, indicating that Cig diversity within the same animal can be high.

Both Cig and Cft colonize the same reptilian hosts (Gilbert et al. 2014a), facilitating potential lateral gene transfer between these two taxa. Indeed, both reptile-associated taxa share specific genomic regions which might confer certain competitive advantages to survive in the reptilian host. Furthermore, similar prophages were identified in the accessory genomes of both taxa, which can serve as a vehicle for novel genetic material and enable gene flow between both taxa. However, no recent recombination events between both taxa were detected in the core genomes. Despite the close genetic relationship and shared host type, the recombination rate between both reptile-associated taxa can be considered low compared with certain other Campylobacter species colonizing a shared host (Sheppard et al. 2008). This could be explained by a recent introduction in the same host or by barriers such as an intrinsic resistance to interspecies recombination or separated niches within the host, as has been shown for C. jejuni (Sheppard et al. 2014). Instead, multiple regions showed higher than expected homology in...
C. iguaniorum, C. hyointestinalis, and members of the C. concisus clade, suggesting gene flow between these distantly related Campylobacter taxa.

Material and Methods

Strains

Characteristics of all strains used in this study are summarized in table 1. Cig strain 1485E (= CCUG 66346 = LMG 28143) was isolated in 2003 from a bearded dragon (Pogona vitticeps) with a hypertrophic and perforated colon. Cig strain 2463D (= CCUG 66347) was isolated in 2003 from a green iguana (Iguana iguana) with chronic interstitial nephritis. All strains were grown on Columbia agar with 5% sheep blood (Oxoid, the Netherlands) in a microaerobic atmosphere (83.3% N₂, 7.1% CO₂, 3.6% H₂, and 6% O₂) at 37 °C for 48 h.

Whole Genome Sequencing

The sequencing of Cig strains 1485E and 2463D was performed using shotgun and paired-end reads obtained on a Roche 454 FLX Genome Sequencer. A total of 292,658 (1485E) or 318,374 (2463D) 454 reads were assembled using the Newbler assembler (v2.6) into single chromosomal scaffolds of 12 (1485E) or 11 (2463D) unique contigs and single megaplasmid scaffolds, providing draft genome sequences with coverages of 68× (1485E) or 65× (2463D). All 454 base calls were validated using 1,570,644 (1485E) or 2,300,370 (2463D) Illumina MiSeq reads, providing an additional 178× (1485E) or 263× (2463D) coverage. Scaffold gaps were filled as described (Merga et al. 2013). Sequences across the contig junctions were confirmed with Sanger sequencing. Assembly was confirmed using PacBio long reads for strain Cig 2463D. PacBio RS reads were assembled into contigs using Quiver (Pacific Bioscience, Menlo Park, CA, USA). Homopolymeric GC tracts were characterized using the high-depth MiSeq reads.

Genome Analysis

Protein-, rRNA-, and tRNA-encoding genes were identified as described (Merga et al. 2013). The 1485E genome was annotated based on Cft strain 03-427T (accession number CP006833) (Gilbert et al. 2013; Fitzgerald et al. 2014) and the annotation of the 2463D genome was based on that of 1485E, with further annotation using Artemis (Rutherford et al. 2000), the identification of Pfam domains (v.27.0) (Finn et al. 2014), and BLASTP comparisons to proteins in the NCBI non-redundant (nr) database. CRISPR regions were identified using CRISPRFinder (Grissa et al. 2007).
Table 1
Features of the *Campylobacter* Strains Used in This Study

| Spedes | Strain | Source organism | Source type | Location | Sequence data | Sequence method | Reference | Accession number |
|--------|--------|-----------------|-------------|----------|---------------|-----------------|-----------|-----------------|
| Cig    | 1485E  | Lizard (Pogona vitticeps) | Feces | NL | WGS, Illumina, PacBio | Gilbert et al. 2014b | CP009043-CP009044 |
| Cig    | 2463D  | Lizard (Iguana iguana) | Feces | NL | WGS, Illumina, PacBio | Gilbert et al. 2015 | CP010995 |
| Cig    | 11502571-1 | Chelonian (Chelodina mccordi) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697811, KU697824, KU697837, KU697850, KU697863, KU697876, KU697889 |
| Cig    | 11502571-4 | Chelonian (Chelodina mccordi) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697812, KU697825, KU697838, KU697851, KU697864, KU697877, KU697890 |
| Cig    | 11502590-1 | Chelonian (Agrionemys horsfieldii) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697813, KU697826, KU697839, KU697852, KU697865, KU697878, KU697891 |
| Cig    | 11502590-4 | Chelonian (Agrionemys horsfieldii) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697814, KU697827, KU697838, KU697851, KU697866, KU697879, KU697892 |
| Cig    | 12501208-3 | Chelonian (Chelonoides carbonaria) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697815, KU697828, KU697840, KU697853, KU697867, KU697880, KU697893 |
| Cig    | 12502279-8 | Chelonian (Aldabrachelys gigantea) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697816, KU697829, KU697841, KU697854, KU697868, KU697881, KU697894 |
| Cig    | 12502285-1 | Chelonian (Aldabrachelys gigantea) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697817, KU697830, KU697843, KU697855, KU697869, KU697882, KU697895 |
| Cig    | 12502360-1 | Lizard (Iguana iguana) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697818, KU697831, KU697844, KU697857, KU697870, KU697883, KU697896 |
| Cig    | 12502824-24 | Chelonian (Aldabrachelys gigantea) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697819, KU697832, KU697845, KU697858, KU697871, KU697884, KU697897 |
| Cig    | 12503949-1 | Lizard (Hemitheconyx caudinctus) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697820, KU697833, KU697846, KU697859, KU697872, KU697885, KU697898 |
| Cig    | 12505338-1 | Chelonian (Stigmochelys pardalis) | Feces | NL | MLST, Sanger | Benejat et al. 2014 | KU697821, KU697834, KU697847, KU697860, KU697873, KU697886, KU697899 |
| Cig    | 13500387-3 | Chelonian (Chelonoides carbonaria) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697822, KU697835, KU697848, KU697861, KU697874, KU697887, KU697900 |
| Cig    | 13500406-3 | Chelonian (Agrionemys horsfieldi) | Feces | NL | MLST, Sanger | Gilbert et al. 2014a | KU697823, KU697836, KU697849, KU697862, KU697875, KU697888, KU697901 |
| Cff    | 82-40   | Human | Blood | US | WGS, Sanger | Perez et al. 1985 | CP000487 |
| Cft    | 03-427  | Human | Blood | US | WGS, Illumina, PacBio | Gilbert et al. 2013 | CP006833 |
| Cft    | SP3     | Snake (Heterodon nasicus) | Feces | UK | WGS, Illumina, PacBio | Gilbert et al. 2016 | CP010953 |
| Cfv    | 97608   | Bovine | Placenta | AR | WGS, Illumina, PacBio | van der Graaf-van Bloois et al. 2014 | CP008810-CP008812 |
| Chh    | LMG 9260 | Human | Feces | BE | WGS, Illumina | Miller et al. 2016b | CP015575 |
| Chi    | CCUG 27631 | Porcine | Gastric biopsy | SE | WGS, Illumina | Miller et al. 2016b | CP015576 |
| Clan   | NCTC 13004 | Human | Feces | SE | WGS, Illumina | Logan et al. 2000 | CP015578 |

Cig, C. iguaniorum; Cff, C. fetus subsp. fetus; Cft, C. fetus subsp. testudinum; Cfv, C. fetus subsp. venerealis; Chh, C. hyointestinalis subsp. hyointestinalis; Chi, C. hyointestinalis subsp. lawsonii; Clan, C. lanienae; AR, Argentina; BE, Belgium; FR, France; NL, Netherlands; SE, Sweden; UK, United Kingdom; US, United States. MLST, multilocus sequence typing; WGS, whole genome sequencing.
complete annotated genome sequence of Cig strain 1485E has been deposited in GenBank under accession numbers CP009043 (chromosome) and CP009044 (megaplasmid) (Gilbert et al. 2014b). The complete annotated genome sequence of the Cig strain 2463D chromosome has been deposited in GenBank under accession number CP010995. Accession numbers of all genomes used in this study can be found in table 1.

The C. iuguanium strain 1485E genome was compared with the genome of strain 2463D and the completed genomes of the closely related taxa C. fetus subsp. testudinum (strains 03-427 and SP3), C. fetus subsp. fetus (strains 82-40), C. fetus subsp. venerealis (strain 97/608), C. hyointestinalis subsp. hyointestinalis (strain LMG 9260), C. hyointestinalis subsp. lawsonii (strain CCUG 27631), and C. lanienae (NCTC 13004). For Campylobacter species outside the C. fetus clade, BLASTP comparisons to proteins in the NCBI non-redundant (nr) database were performed. A local BLAST was performed based on the predicted proteomes of all genomes and the results were screened for features specific for one or both Cig strains and the other members of the C. fetus clade. Using JSpecies v.1.2.1 (Richter and Rosselló-Móra 2009) and BLAST v.2.2.26, average nucleotide identity (ANI) values based on the whole genome sequences were calculated for these strains as a measure of genetic relatedness. The BLAST parameters were: X = 150, q = -1, F = F, e = 1e-15, and a = 2. To visualize genomic regions specific for Cig, the BLAST ring image generator (BRIG) (Alikhan et al. 2011) was used, based on BLAST v.2.2.26.

**MLST**

Multilocus sequence typing (MLST) was performed for all Cig strains listed in table 1. The MLST loci were extracted from the genomes of 1485E and 2463D and sequenced as described previously for the other Cig isolates (Miller et al. 2012). For HFglt, the annealing temperature was lowered to 46 °C and 40 instead of 35 cycles were used. Sequences were trimmed and concatenated. In MEGA v.6.06, the concatenated sequences were aligned using MUSCLE and a Maximum likelihood dendrogram was created using 500 bootstraps. Accession numbers of the MLST sequence data can be found in table 1.

**Supplementary Material**

Supplementary tables S1–S4 and figure S1 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org).

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