# Phenotypic and genetic analyses of two *Campylobacter fetus* isolates from a patient with relapsed prosthetic valve endocarditis

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## Abstract

*Campylobacter fetus* can cause intestinal and systemic disease in humans and are well established veterinary and economic pathogens. We report the complete genomic sequences of two *C. fetus* subsp. *fetus* (Cff) isolates recovered in 2017 (CITCf01) and 2018 (CITCf02) from a case of recurrent prosthetic valve endocarditis. Both were capable of growth aerobically. Their genomes were found to be highly conserved and syntenic with 99.97% average nucleotide identity (ANI) while differences in their respective *sap* loci defined the temporal separation of their genomes. Based on core genome phylogeny and ANI of 83 Cff genomes belonging to the previously described human-associated Cff lineage, CITCf01 and CITCf02 grouped in a clade of eleven sequence type (ST)3 Cff (including the Cff type strain NCTC 10842$^{T}$). CITCf01 and CITCf02 were marked for their lack of unique genomic features when compared to isolates within the subspecies and the type strain in particular. We identified point mutations in oxidative stress response genes, among others, that may contribute to aerobiosis. We report a case of Cff causing relapsed prosthetic valve endocarditis and we highlight the *sap* island as a polymorphic site within the genetically stable ST3 lineage, central to pathogenicity.

## Introduction

*Campylobacter fetus* is a well-established veterinary and economic pathogen capable of causing intestinal and systemic disease in humans. Among the three currently defined *C. fetus* subspecies, *Campylobacter fetus* subsp. *venerealis* (Cfv) is a bovine-adapted clone causing bovine genital campylobacteriosis and infectious infertility in cattle but is an infrequent cause of human infection (Wagenaar *et al.* 2014) *Campylobacter fetus* subsp. *testudinum* (Cft) is largely reptile-associated (Fitzgerald *et al.* 2014) though the type strain (Cft strain 03-427) was originally isolated from human blood in 2003, while the subspecies was not defined until 2014 (Fitzgerald *et al.* 2014).

The generalist lineage of *Campylobacter fetus* subsp. *fetus* (Cff) colonises the intestinal and genital tract of ovine, bovine, avian and human hosts (Iraola *et al.* 2017) and is associated with epizootic septic abortion in ruminants, predominantly sheep (Sahin *et al.*). Cff is also a cause of intestinal illness in humans (Bullman *et al.* 2011) but notably is the most frequent *Campylobacter* causing bacteraemia (Wagenaar *et al.* 2014). Cff has been well documented to cause a spectrum of systemic illnesses in immunocompromised humans with a tropism for the vascular endothelium, especially at sites of pre-existing damage (Morrison *et al.* 1990). Reported infections include but are not limited to; septic abortion (Hood and Todd 1960), spondylodiscitis (Cunha *et al.* 2021), outbreaks of nosocomial neonatal meningitis (Morooka *et al.* 1988), peritonitis (Wens *et al.* 1985), soft tissue infections (La Scola, Chambourlier and Bouillot 1998), endocarditis (Durovic *et al.* 2021) and other vascular pathologies (vasculitis, pericarditis, thrombophlebitis, mycotic aneurysms, aortitis among others) (Morrison *et al.* 1990; Wagenaar *et al.* 2014; Nulens *et al.* 2018; Eke, Doub and Chua 2021). Persistent (Nakazawa *et al.* 2018), recurrent or relapsed (Tremblay, Gaudreau and Lorange 2003; Tu, Gaudreau and Blaser 2005; Nishikubo *et al.* 2021) and multifocal invasive *C. fetus* infections (Durovic *et al.* 2021) have also been reported. In some instances, systemic *C. fetus* infections have been recorded in immunocompetent individuals (Gazaigne *et al.* 2008) or were not preceded by diarrhoeal illness (or Cff was not detected from faeces) (Eke, Doub and Chua 2021). The source of infection is often linked to animal exposure (Wagenaar *et al.* 2014; Nakazawa *et al.* 2018) or the consumption of contaminated meat products.

Kiggins and Plastridge (1956) reported that atmospheric conditions of 5% oxygen and 10% carbon dioxide were optimal for *C. fetus* and growth in air is not a species trait (Véron and Chatelain 1973). In nature, or during infection, *Campylobacter* spp. are exposed to iron limiting conditions and oxidative stress. Aerotolerant *Campylobacter* spp. have been described in the literature, with varying degrees of survival and growth in air, typically *Campylobacter jejuni* isolated from retail meat or food animals (Oh, McMullen and Jeon 2015; O'Kane and Connerton 2017; Kim *et al.* 2019) or humans (Rodrigues *et al.* 2015). Reports of Cff aerobiosis are limited. Neill *et al.* (1985) described rouge *C. fetus* strains that grew aerobically and failed to reduce nitrate and due to the complexity and recent expansion of the genus, these isolates may belong to a different species/subspecies category.

In this paper, we describe two Cff isolates (CITCf01 and CITCf02) recovered in 2017 and 2018, respectively from blood cultures of an Irish patient with recurrent prosthetic valve endocarditis, that were marked for their ability to grow aerobically. Reports of *C. fetus* causing prosthetic valve endocarditis are limited (Caramelli *et al.* 1988; Farrugia, Eykyn and Smyth 1994; Peetermans *et al.* 2000; Haruyama *et al.* 2011; Reid *et al.* 2016; Petridou, Strakova and Simpson 2018). In this study, our aim was to investigate the phenotypic characteristics, antimicrobial resistance (AMR), genomic composition and phylogeny of such isolates.

## Methodology

### Bacterial isolates and growth conditions

Clinical Cff isolates CITCf01 and CITCf02 were obtained from an elderly male patient (77 years upon first isolation) in the Mercy University Hospital, Cork, Ireland in a case of recurrent prosthetic valve endocarditis, where CITCf01 was isolated from blood cultures in 2017 and CITCf02 in November 2018.

The Cff isolates were recovered from aerobic and anaerobic blood culture bottles (BacT/ALERT FA Plus and FN Plus) on the BacT/ALERT Virtuo continuous monitoring blood culture system (bioMérieux, Marcy-l'Étoile, France). The Cff type strain NCTC 10842$^{T}$ (hereafter referred to as NCTC 10842$^{T}$) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ): DSM 5361. Isolates were subsequently maintained at –80°C in defibrinated horse blood (Oxoid) with 30% (vol/vol) glycerol and recovered from frozen stocks on Columbia blood agar (CBA) (Fannin, Dublin, Ireland) and incubated for 22 ± 2/44 ± 4 hours at 37°C, under microaerobic conditions, and sub-cultured.

### Identification and subspeciation

Cff isolates CITCf01 and CITCf02 were identified and subspeciated using the NH ID card on the analytical profile index (API) of the VITEK 2 (bioMérieux, Marcy-l'Étoile, France) according to manufacturer's instructions, and as described previously (Lynch *et al.* 2019). Species identification was performed in duplicate by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) on the Bruker MALDI Microflex Biotyper instrument (Bruker Daltonics, Billerica, MA, USA), as described previously (Koziel *et al.* 2014) and detailed in the Supplementary Methods section.

### Biochemical and phenotypic profiling

To characterise isolates CITCf01 and CITCf02, classical phenotypic and biochemical testing was performed as previously described (Sandstedt and Ursing 1991; On and Holmes 1992; Ursing, Lior and Owen 1994; Cowan 2012) with appropriate control organisms for each test (Cowan 2012). Protocols for each test are provided in the Supplementary Methods section.

### Phenotypic antimicrobial resistance profiling

Isolates CITCf01 and CITCf02 were tested for resistance to five clinically relevant antimicrobials namely, tetracycline (0.5-64 µg/mL), erythromycin (1-128 µg/mL), ciprofloxacin (0.12-16 µg/mL), gentamicin (0.12-16 µg/mL), and streptomycin (0.25-16 µg/mL) using broth microdilution minimum inhibitory concentration (MIC) testing according to ISO 20776:2006 with EUCAMP2 Sensititre plates (Thermo Fisher Scientific, Waltham, MA, USA) - described in the Supplementary Methods section.

### Whole-genome sequencing

Whole-genome sequencing (WGS) of CITCf01 and CITCf02 was performed using both Illumina and Oxford Nanopore sequencing to obtain short and long sequence reads. Hybrid genome assembly was performed using Unicycler v0.4.0 (Wick *et al.* 2017). Full details of DNA extraction, library preparation and sequencing are provided in the Supplementary Methods section.

**Bioinformatic analysis of CITCf01 and CITCf02 and comparison to the *Campylobacter fetus* subsp. *fetus* type strain NCTC 10842$^{T}$**

Predicted open reading frames (ORFs) were annotated with Prokka 1.13.5 (Seemann 2014), using a reference annotation database derived from Cff (GenBank accession numbers: CP008808.1, CP000487.1, LS483431.1), Cfv (GenBank accession number: CM001228.1) and Cft (GenBank accession number: CP010953.1) genomes. Additional functional annotation tools used are listed in the Supplementary Methods section. AMR genes were screened using the Resfinder database (Zankari *et al.* 2012) on ABRicate (https://github.com/tseemann/abricate) and the Comprehensive Antibiotic Resistance Database (Jia *et al.* 2017).

Clustering of gene orthologues in CITCf01 or CITCf02 and NCTC 10842$^{T}$ was performed using ProteinOrtho [v. 6.0.14] (Lechner *et al.* 2011) with the DIAMOND (Buchfink, Xie and Huson 2014) alignment algorithm. Core and accessory gene counts were determined with 95% minimal sequence similarity, 80% minimal coverage and 60% minimal percent identity. Genome map was generated using CGView (Grant and Stothard 2008). Single nucleotide polymorphism (SNP) and indel (small insertion or deletion) calling were performed with Snippy [v. 4.5.0] (https://github.com/tseemann/snippy) using default parameters, mapping the paired-end reads of CITCf01 to NCTC 10842$^{T}$ as the reference genome, or paired-end reads of CITCf02 to CITCf01 as the reference genome. Nonsynonymous SNPs were called using Mummer using default parameters [v. 4.0.0beta2] (Marçais *et al.* 2018). Further annotative and comparative tools used are listed in the Supplementary Methods section.

***Campylobacter fetus* subsp. *fetus* phylogeny and pangenome analysis**

Additional complete and draft Cff genomes (*n*=81) were retrieved from NCBI and included 73 human-associated *C. fetus* genomes collected in a metagenomics study by Iraola *et al.* (2017). Isolate attributes are detailed in Supplementary information 1, Table S1. Paired-end reads were assembled using SPAdes v.3.11.1 and quality checked using fastqc. Pairwise average nucleotide identity (ANI) was calculated using FastANI (Jain *et al.* 2018) and the Cff ANI heatmap was created using Pheatmap v1.0.12 (Kolde 2012). Core and accessory genomes of the 83 (total) Cff genomes were analysed using Roary [v. 3.12.0] (Page *et al.* 2015). Coding sequences (CDS) with at least 95% identity were considered as part of the core genome for the analysis, and core is defined as a gene present in at least 99% of samples (van der Graaf-Van Bloois *et al.* 2014; Iraola *et al.* 2017). Concatenated core genes and their subsequent alignment was performed by Roary with the construction of a maximum-likelihood phylogenetic tree performed by FASTTREE [v. 2.1.10] (Price, Dehal and Arkin 2010). The phylogenetic tree was visualised using iTOL v5 (Letunic and Bork 2016). Multi locus sequence types (MLST) profiles were interrogated using the PubMLST *Campylobacter* database (Jolley, Bray and Maiden 2018). Bayesian-clustering was also performed on Roary concatenated core genes (Cheng *et al.* 2013). CRISPR (clustered regularly interspaced short palindromic repeats) spacer typing was used to differentiate the ST3 Cff isolates. CRISPR spacer sequences were extracted with MinCED [v.0.4.2] (Bland *et al.* 2007) and redundant sequences were removed using BLASTn (-task BLASTn-short). The R package heatmaply was used for hierarchical clustering of shared CRISPR sequences and visualisation (Galili *et al.* 2018).

**Data availability**

Genomes for both Cff isolates CITCf01 and CITCf02 were deposited in GenBank under the following accession numbers CP072664 and CP072665.

## RESULTS

### Isolation and identification

We report the isolation, phenotypic behaviour and genomic attributes of two Cff isolates recovered from an elderly male patient in a case of recurrent prosthetic valve endocarditis. Cff isolates CITCf01 and CITCf02 were isolated from blood culture bottles in 2017 and 2018. Although stool samples were not taken on these occasions, *Campylobacter* was not detected in a stool sample taken in April 2017. A reliable identification (MALDI score > 2.000) of Cff subspecies membership was made for the sister isolates CITCf01 and CITCf02, corresponding to their VITEK profile (bionumber: 0210000522).

### Phenotypic, biochemical characteristics and antimicrobial susceptibility of CITCf01 and CITCf02

Cff isolates CITCf01 and CITCf02 behaved as typical Cff (Table 1), despite their unusual ability to grow under aerobic conditions on CBA, not observed for NCTC 10842$^T$, as confirmed in this study. Faint growth of isolates CITCf01 and CITCf02 appeared after 24 hours incubation and by 48-72 hours, colonies were coarser and smaller than their counterparts grown under microaerobic conditions. Aerobic cultures of CITCf01 and CITCf02 rarely grew beyond the third quadrant of the CBA plate after 72 hours of incubation. The isolates also grew aerobically on Mueller-Hinton agar, although weaker than on CBA.

Both clinical Cff isolates CITCf01 and CITCf02 were sensitive to tetracycline, erythromycin, ciprofloxacin, gentamicin and streptomycin (Table 2). CITCf01 and CITCf02 were resistant to nalidixic acid (30 µg) and susceptible to cephalothin (30 µg) – where resistance to nalidixic acid is a *C. fetus* specific trait (Gazaigne *et al.* 2008; Cowan 2012). Resistance to antimicrobials is typically lower within the species compared to *C. jejuni* and *Campylobacter coli* (Pacanowski *et al.* 2008). Third generation cephalosporins, fluoroquinolones and macrolides are not indicated as empirical treatment of *C. fetus* bacteraemia or invasive infections due to increasing rates of resistance (Gazaigne *et al.* 2008; Lee *et al.* 2011; Mosca, Del Gaudio and Miragliotta 2017; Nulens *et al.* 2018), multidrug resistance (Anstead *et al.* 2001) or post-treatment ciprofloxacin resistance in *C. fetus* (Meier *et al.* 1998; Nishikubo *et al.* 2021). A carbapenem or amoxicillin–clavulanate in monotherapy, or in combination with gentamicin, is the current practice as first-line therapy, although an *in vitro* imipenem-resistant *C. fetus* has been reported (Nulens *et al.* 2018). Notably, a gentamicin-resistant *C. fetus* isolate was recovered from retail pork in Ireland over ten years ago (Scanlon *et al.* 2013). Correct early antimicrobial therapy improves patient outcomes (Morrison *et al.* 1990), but despite increasing rates of AMR within the genus relatively little is known about the true rates of resistance among *C. fetus* isolates, accounting for geography and host (Kwon *et al.* 1994; Tremblay, Gaudreau and Lorange 2003).

### Genomic features of Cff isolates and comparative analysis

Genome assembly was performed using short- and long-reads to obtain high-quality complete genome sequences for Cff sister isolates CIT01 (recovered in 2017) and CIT02 (recovered in 2019) with 334x and 688x coverage, respectively. The salient properties (genome size, % GC-content, CDS and tRNA number) of their genomes were found not to be appreciably different from the type strain NCTC 10842$^T$ (Table 3 and Supplementary information 2, Figure S1), or that reported for the species in general (Oliveira *et al.* 2016; Hou *et al.* 2018; Miller and Yee 2019). No obvious antimicrobial resistance determinants among predicted among their genes. Furthermore, no plasmids were identified among genomes assemblies of isolates. Three prophage elements (5.8 kbp, 9.6 kbp and 5.6 kbp in size) were found shared among genomes with similar elements also associated with the genome of NCTC 10842$^T$.

The position of Cff isolates CITCf01 and CITCf02 within the major human *C. fetus* lineage was determined by comparison to 81 non-redundant genomes of Cff obtained from public repositories (details of genomes can be found in Supplementary information 1, Table S1). There are two major *C. fetus* lineages (bovine- and human-associated), described previously (Iraola *et al.* 2017; Abdel-Glil *et al.* 2020) and the majority of selected genomes in this represent Cff isolated from human sources and the four nonhuman isolates belong to human *C. fetus* lineage (Abdel-Glil *et al.* 2020). An ANI of >99.2% was found to be shared among all genomes showing that the isolates reported in the study are highly conserved at the nucleotide level (Supplementary information 2, Figure S2), even among isolates obtained from different sources (human, bovine and ovine) and originating from ten countries, consistent with previous reports (van der Graaf-van Bloois *et al.* 2014; Escher *et al*., 2016).

Multi-locus sequencing typing (MLST) is a common methodology for *C. fetus* subtyping, with at least 73 deposited STs on PubMLST to date. Phylogenetic placement utilising shared genes (core genes) was conducted with CITCf01 and CITCf02 among the representative 81 Cff genomes - comprised of eight defined sequence types (STs). Isolates CITCf01, CITCf02 along with NCTC $10842^{T}$ could be seen to cluster with nine Cff isolates with an >99.9% ANI (Figure 1, Supplementary information 2, Figure S2), where the genomes of CITCf01 and CITCf01 share 99.97% ANI. Isolates in this ST3 cluster (*n*=11) are from the United Kingdom (UK), France and Canada, with nine in total being isolated from humans and two from ovine hosts. MLST clustering of Cff genomes corresponded with core genome phylogeny (Figure 1), similar to findings in other studies (van der Graaf-van Bloois *et al.* 2014; Iraola *et al.* 2017; Abdel-Glil *et al.* 2020). BAPS identified seven clusters among the Cff genomes and was consistent with the observed phylogenetic clusters. ST3 represents a generalist lineage where isolates have been recovered from human, ovine, bovine, equine and simian hosts (Van Bergen *et al.* 2005; Escher *et al.* 2016; van der Graaf-van Bloois *et al.* 2016; Emele *et al.* 2019). ST3 has been reported as one of the most frequently isolated STs from humans, sheep and cattle in the UK (Duncan *et al.* 2014). ST4 is dominant among bovid-associated *C. fetus*, but members of both major lineages (human and bovine) belong to the ST (Iraola *et al.* 2017; Abdel-Glil *et al.* 2020).

Pangenome analysis was performed to investigate the genetic diversity of CITCf01 and CITCf02 within the context of the subspecies, among the above mentioned 81 Cff genomes. These genomes were predicted to contain a median of 1,756 ORFs (range 1,738-1,941) that could be placed into a total pangenome of 2,899 orthologous groups (OGs). The core genome was found to be comprised of 1,447 genes (approximately 83% of each genome), an accessory genome with a median of 279 ORFs (range 261-464) and unique genes per strain with a median of 2 ORFs (range 0-73). The genomes of Cff isolates CITCf01 and CITCf02 were particularly marked for the lack of novel genetic elements when compared to other members of the human *C. fetus* lineage. The genes associated with CITCf01 and CITCf02 being comprised of those identified as core genes of Cff, with an accessory genome of 270 or 280 ORFs

Further typing based on CRISPR arrays was performed to understand the origin and divergence of CITCf01 and CITCf01 among nine closely related Cff genomes. CRISPR-Cas systems direct RNA interference-like cleavage of foreign DNA (phage, plasmids, etc.) and form part of the bacterial adaptive immune system (Soto-Perez *et al*. 2019). These systems function by storing sequences of foreign DNA from past exposures as spacers that form part of CRISPR arrays. The acquisition of new spacers to the CRISPR array occurs systematically, with the addition of new spacers at the 5' end of an array, with occasional sporadic loss of spacers (Barrangou *et al.* 2007). However, the functionality of CRISPR-Cas systems in *C. fetus* has yet to be demonstrated (Calleros *et al.* 2017), but may contribute to the stabilisation of the subspecies (Ali *et al.* 2012). Based on the concept that these arrays undergo expansion/deletion of spacer elements; it is possible to determine the divergence of closely related strains. Cff isolates CITCf01 and CITCf02 harbour two CRISPR arrays (arbitrarily named CRISPR array-I and CRISPR array-II, in order of appearance on the genome). These arrays are composed of an identical 30 bp direct repeat sequence (GTTTGCTAATGACAATGTTTGTGTTGAAAC) and 8 and 26 spacers (9 and 27 repeat units), respectively, with similar arrays found among the other Cff isolates. The genomes of the eleven strains possess a median of 41 spacers (range 20 to 62) comprising of 72 unique spacers. Hierarchically clustering of the presence/absence of unique spacers among genomes shows that Cff

isolates with different origins will tend to differ with the spacer content of their CRISPR arrays (Calleros *et al.* 2017). Additionally, analysis shows that isolates CITCf01 and CITCf02 share similar CRISPR spacers, supporting the notion that they are the same isolate, obtained at different time points (Supplementary information 2, Figure S3). Alignment of their CRISPR arrays also verifies this observation with arrays sharing the same arrangement of their spacers. Furthermore, spacer content of CITCf01 and CITCf02 was most similar to genomes of ERS672259 and ERS672260, both isolated in France from human sources.

**Genetic factors potentially contributing to aerobic growth**

We took advantage of the high degree of genetic relatedness between Cff isolates CITCf01, CITCf02 and the subspecies type strain NCTC $10842^{T}$ to determine any changes at a genetic level that may account for their differential survival in air. Considering the lack of large insertions or deletions, major gene rearrangements or the acquisition of novel genes, we believe subtle differences in regulation of genes involved in oxidative stress may be responsible. A total of 155 and 170 SNPs and indels were detected on the genomes of CITCf01 and CITCf02, respectively when compared to NCTC $10842^{T}$. Nonsynonymous mutations were detected in a subset of 60 ORFs (outside of the *sap* island) and KEGG functional categories were assigned to 76.67% of sequences which were predominantly associated with signal transduction, genetic regulation, cellular processes, carbohydrate and amino acid metabolism (Supplementary information 2, Figure S4).

A high proportion of these nonsynonymous mutations were located in a 50 kbp region containing various oxidative stress response genes. We detected an interrupted 4Fe-4S ferredoxin gene (corresponding to *fdxB*) within this region (Figure 2) in both isolates and *fdxB* was recently reported to be upregulated upon exposure to bile in *C. jejuni* (Kreuder *et al.* 2017). Ferredoxins function as electron carriers in a wide variety of a biological reactions (Bruschi and Guerlesquin 1988) and iron-sulphur clusters have functions relating to electron transfer, gene regulation, environmental sensing and substrate activation (Frazzon, Fick and Dean 2002). A putative iron-inducible 2[4Fe-4S] ferredoxin *fdxA* gene was also identified within this region; previously described as a central player in the aerotolerance of *C. jejuni* (Vliet *et al.* 2002). A mutated homeostatic response regulator transcription factor (*hsrA*) and HAMP domain-containing histidine kinase genes are located directly upstream of a truncated ferredoxin gene (*fdxB*). HsrA is known to mediate oxidative stress response as part of a two-component signalling system in *H. pylori* (Olekhnovich *et al.* 2014; Flint, Stintzi and Saraiva 2016) and *C. jejuni* (Garénaux *et al.* 2008). Garénaux *et al.* (2008) reported the upregulation of a HsrA homologue in *C. jejuni* in response to oxidative stress.

Guanosine-5'-triphosphate,3'-diphosphate pyrophosphatase *gppA* is located directly downstream of *fdxB* and GppA catalyses the conversion of guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp), where the latter is an alarmone regulating bacterial cellular activities in response to oxidative, amino acid or stringent stress response (Keasling, Bertsch and Kornberg 1993; Kumar *et al.* 2016; Ronneau and Hallez 2019). Another putative GppA with higher homology to SpoT (known to maintain (p)ppGpp levels in *C. jejuni*) is located elsewhere on the genomes of CITCf01 and CITCf02 (J5248_01174 and J5249_01171). Alkyl hydroperoxide reductase C (*ahpC*), peroxide response transcriptional regulator (*perR*) and a further downstream iron ABC transporter operon are also located within this region (Figure 2), previously reported as important oxidative stress response factors that contribute to aerotolerance in *C. jejuni* (Baillon *et al.* 1999; Palyada, Threadgill and Stintzi 2004; Handley *et al.* 2015; Rodrigues *et al.* 2016).

Point mutations were detected in multiple transcription factors genes throughout the genomes of CITCf01 and CITCf02 (Supplementary information 1, Table S2). A sole LysR-type regulator was shown to have a profound effect on the global stress response and energy metabolism in *C. jejuni* (Dufour *et al.* 2013). Of note, a C115T transition mutation (Pro-39-Ser) was detected in one copy of an encoded Rrf2 family transcriptional regulator gene and homologues described in *Escherichia coli* regulate the transcription of several operons and genes involved in the biogenesis of Fe-S clusters and Fe-S-containing proteins (Yeo *et al.* 2006). A T236C transition mutation (Val-79-Ala) was detected in a TetR/AcrR family transcriptional regulator gene (corresponding to the DNA-binding homeobox

(InterPro accession number: IPR009057)) (Kisker *et al.* 1995; Mannervik 1999), located downstream of an iron uptake ABC transporter complex and cytochrome c oxidase accessory protein gene (*ccoG*) and upstream of an iron-inducible, apo-Fur-repressed hydrogenase (Flint, Stintzi and Saraiva 2016), which may play a role in iron scavenging and oxidative stress response (Rodrigues *et al.* 2016; Liu *et al.* 2018).

Much of the data surrounding stress response in *Campylobacter* surrounds *C. jejuni* (Takata *et al.* 1995; Park 2002; Rodrigues *et al.* 2016; Teren *et al.* 2019; Song *et al.* 2020) Transcriptome studies in *C. jejuni* point to a complex approach to stress and aerobic response in *Campylobacter* (Rodrigues *et al.* 2016; Kreuder *et al.* 2017). The overlap between oxidative stress response and general stress response (Birk *et al.* 2012; Varsaki *et al.* 2015) is thought to be attributed to altered metabolism during stress and disturbed electron transport systems leading to the generation of reactive oxygen species, thus oxidative stress responses promote homeostasis (Kim *et al.* 2015). Although we have identified some differences in genetic content between Cff isolates CITCf01, CITCf02 and NCTC 10842$^{T}$, gene expression studies are necessary to determine factors contributing to aerobic growth in Cff. Certainly, other Cff isolates should be tested for their ability to grow aerobically (given 72 hours growth at 37°C to observe slow growth) to assess if this is more common than originally thought, as this has implications for the survival and evolution of the subspecies.

**The *sap* island differentiates CITCf01 and CITCf02**

The genomes of CITCf01 and CITCf02 are highly conserved and syntenic, where a total of 18 SNPs and indels were detected between them. However, the isolates were readily distinguished by their respective *sapB* genomic islands. This gene cluster is associated with the proteinaceous S-layer on the outer surface of the bacterial cell - composed of a monolayer of regularly spaced and self-assembling paracrystalline S-layer proteins (SLPs) (Sleytr and Messner 1983; Koval 1988) and is common to just two *Campylobacter* species, namely *C. fetus* and *Campylobacter rectus*. Sap homologues, promoter, transcriptional terminators sequences and SLP secretion systems are located in the invertible *sap* island (Tu, Hui and Blaser 2004) and the *sap* homologue located directly downstream of the *sap* promoter is transcribed, while all other *sap* homologues are transcriptionally silent (Tu, Gaudreau and Blaser 2005). The conserved 5' *sapA* region mediates recombination and inversion among multiple homologues, resulting in phase variation (Fagan and Fairweather 2014). *C. fetus* serotypes A and B correspond to SapA and SapB homologues; differentiated by their respective 183 N-terminal amino acid residues (Dworkin, Tummuru and Blaser 1995).

The *sap* island present in CITCf01 spans 75 kbp and contains eleven full (2415 to 3882 bp) and two truncated *sapB* homologues. While the CITCf02 *sap* island is considerably shorter (60 kbp) with seven *sapB* homologues and eight partials - likely reflecting recombination remnants (Figure 3). A putative Shine-Dalgarno sequence (AGGAG) (Tu, Gaudreau and Blaser 2005) precedes (-10 bp) of CITCf01 and CITCf02 *sapB* homologues but is not present upstream of the partial *sapB* homologues in CITCf01. GDP-mannose 4,6-dehydratase (*wcbK*) (J5248_00491 and J5249_00488) has been exclusively associated with Cff SapB strains (Kienesberger *et al.* 2014) and was found to be located at the 3' end of the *sap* loci in isolates CITCf01 and CITCf02 (Figure 3). Genetic rearrangements of the *sap* island promote long term colonisation of the host (Tu, Gaudreau and Blaser 2005).

*sapDEF* encodes a type I secretion system that has been recognised as the constitutive transporter required for SLP secretion to the cell surface (Thompson *et al.* 1998; Thompson 2002). We identified *sapDEF* homologues on the genome of CITCf01, but these genes are absent in CITCf02 and NCTC 10842$^{T}$. Similarly, *sapDEF* homologues were detected in 95.3% (*n*=81) of Cff isolates examined in this study, while the predicted MlaFEDB-like transporter apparatus, also located within the *sap* island, was detected in all Cff genomes examined. The Gram negative MlaFEDB complex is known to function as an intermembrane phospholipid trafficking system, preventing their accumulation in the outer membrane (Malinverni and Silhavy 2009; Chi *et al.* 2020). Considering the absence of *sapDEF* on the genome of CITCf02, it's likely another transport system facilitates or compensates for the transport of SLPs to the cell surface. Recently, the presence of *sapDEF* and the absence of known *sap*

homologues was reported in a putative novel species "*Candidatus* Campylobacter infans"; phylogenetically related to *C. fetus* (Bian *et al.* 2020).

## DISCUSSION

We report the isolation, phenotypic behaviour and genomic attributes of two Cff isolates recovered from an elderly male patient in a case of recurrent prosthetic valve endocarditis. *C. fetus* bloodstream infections are typically seen in elderly (often male) or immunocompromised populations (Gazaigne *et al.* 2008; Pacanowski *et al.* 2008; Cypierre *et al.* 2014; Wagenaar *et al.* 2014; Patrick *et al.* 2018).

The complete picture of the adaptive strategies employed by Cff isolates CITCf01 and CITCf02 to grow under an ambient gaseous atmosphere remain unclear, but it appears that subtle genetic changes are responsible. The ability to grow in air should remain an important consideration in the transmission of Cff in the environment - clinically or agriculturally. The unexpected ability of isolates in this study to grow under aerobic conditions may have promoted survival in the open environment or the successful establishment of infection *in vivo*. Aerotolerance and hyper-aerotolerance have been associated with increased resistance to oxidative stress (peroxide and superoxide) in *C. jejuni* and *C. coli* strains (Oh, McMullen and Jeon 2015; O'Kane and Connerton 2017). Despite the frequent exposure of obligate microaerobic *Campylobacter* spp. to oxidative stress in the environment or during colonisation and infection, the adaptive strategies used to survive and even grow in atmospheric oxygen remain poorly understood (Kumar *et al.* 2016). Campylobacters lack many classical stress response mechanisms, contributing to their growth under narrow temperature ranges and general inability to grow under aerobic conditions (Kumar *et al.* 2016). However, a consideration of the high level of conservation of CITCf01 and CITCf02 to other isolates of Cff in relation to their genomic composition (nucleotide homology, shared genes, etc.) shows that they are not atypical isolates of this subspecies, unless this phenotype is a result of SNPs or indels among shared genes associated with signal transduction, genetic regulation, cellular processes, carbohydrate and amino acid metabolism.

*C. fetus* evades antibody-mediated death via high frequency antigenic variation of the S-layer by recombination of *sap* homologues (Thompson 2002). Our findings highlight this feature of the polymorphic *sap* island to facilitate recurrent systemic Cff infection, observed as a hotspot for inversion and deletions of genes.

We also show conservation among ST3 Cff strains, pointing to a stable lineage with CITCf01 and CITCf02 belonging to this ST. Limited source attribution of sporadic cases exists, but food animals are often suspected (Costa *et al.* 2020), despite the infrequent isolation of Cff from food (Scanlon *et al.* 2013; Wagenaar *et al.* 2014), where the latter may be a consequence of insufficient detection systems. Undercooked meat was implicated as the likely cause of *C. fetus* bacteraemia and meningitis in a 33-year-old woman on maintenance therapy for acute lymphoblastic leukaemia, suggesting that neutropenic diet should be considered (Nakatani *et al.* 2021). As an alternative view to that of Nakatani *et al.* (2021), *C. fetus* can establish as a member of the intestinal microbiota of healthy individuals (Iraola *et al.* 2017), with the human gut serving as a natural reservoir. It is difficult to make this assumption about this case, as stool samples were not taken on the patient when CITCf01 and CITCf02 were isolated and *Campylobacter* spp. was not detected from a subsequent stool sample taken in the intervening period and several blood cultures from the patient yielded no growth in late 2018, 2019 and 2020. Pertinent to the current paper, the multiplex PCR assay used to detect enteric pathogens in a large number of Irish diagnostic laboratories targets *C. jejuni* and *C. coli* and not the *Campylobacter* genus. It is possible that the human gut acted as a reservoir for this bacterium before causing relapsed infective prosthetic valve endocarditis. Long-term endogenous persistence or intracellular survival of *Campylobacter* spp. in the human host may not be surprising (Casey *et al.* 2017), considering this tendency among other curved or spiral bacterial genera.

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## Conflicts of Interest

The authors declare no conflict of interest.

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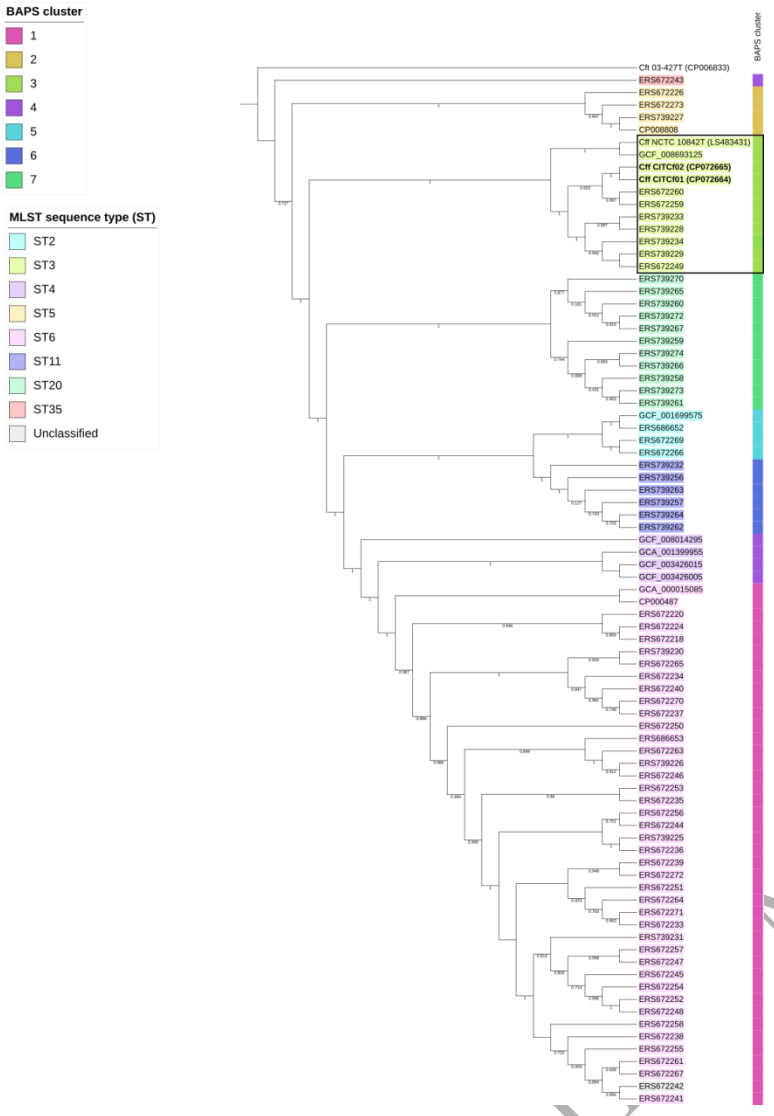


**Figure 1.** Maximum-likelihood phylogenomic tree based on core genome alignment of 83 *Campylobacter fetus* subsp. *fetus* (Cff) isolates. *C. fetus* subsp. *testudinum* (Cft) type strain 03-427$^{T}$ (GenBank accession number: CP006833.1) was used as the outgroup. Branches are labelled with sequence accession numbers and isolate details are listed in Table S1. The vertical colour strip shows BAPS clusters. Branch labels are shaded with corresponding multi-locus sequence type (ST). Bootstrap values (0-1) based on 500 repetitions and are shown beside branches. Cff isolates CITCf01, CITCf02 and type strain NCTC 10842$^{T}$ are labelled on the tree and cluster with ST3 Cff genomes, highlighted in an open black box.

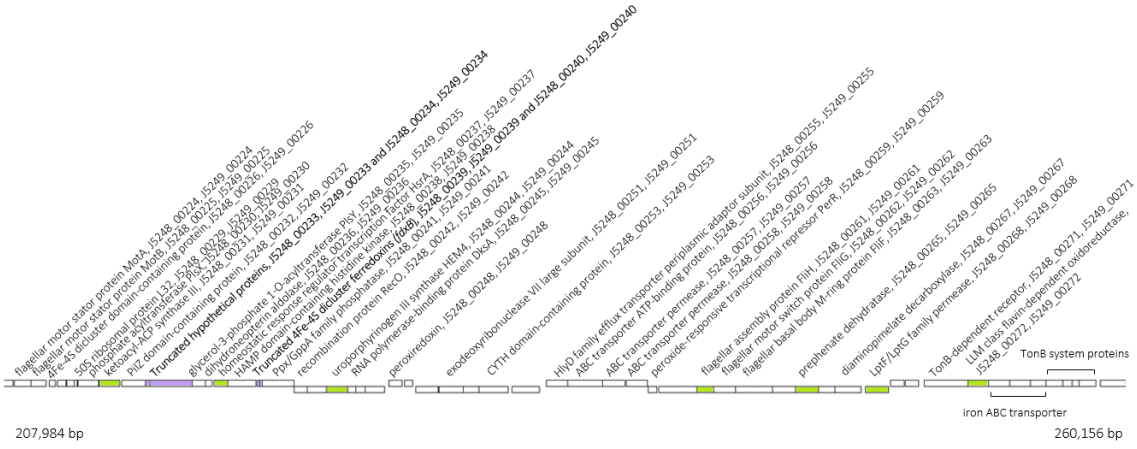


**Figure 2.** Genomic island of *Campylobacter fetus* subsp. *fetus* isolate CITCf01 (mirrored in isolate CITCf02) harbouring a high abundance of genes putatively associated with oxidative stress response. Open reading frames harbouring point mutations resulting in amino acid substitutions are highlighted in green. The truncated genes are highlighted in purple.

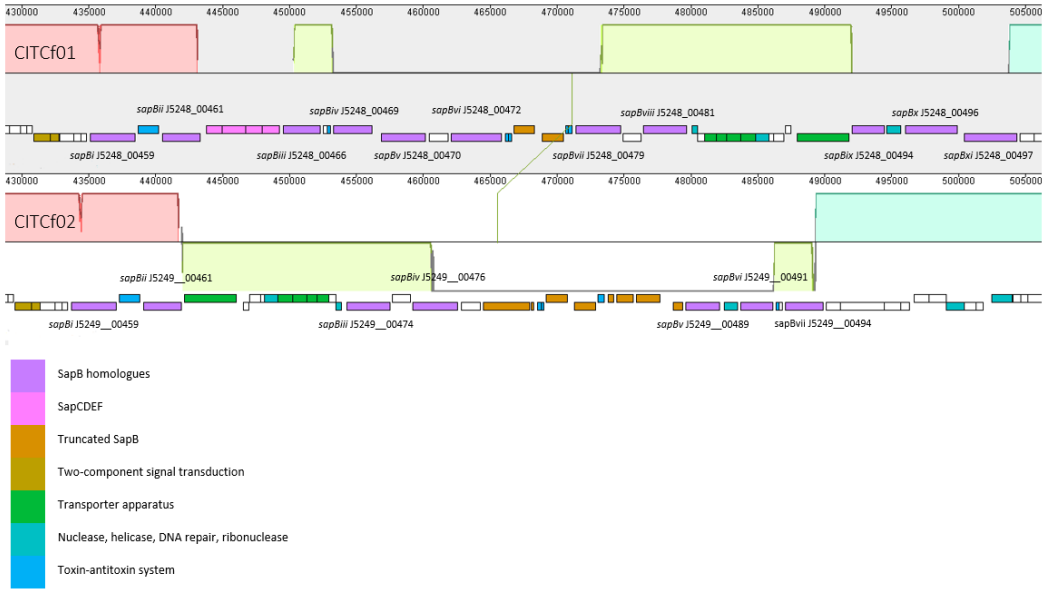


**Figure 3.** Genomic arrangement of *Campylobacter fetus* subsp. *fetus* isolates CITCf01 and CITCf02 chromosomal *sap* islands, created using Mauve.

**Table 1.** Phenotypic characteristics of *Campylobacter fetus* subsp. *fetus* (Cff) isolates CITCf01 and CITCf02 compared to *C. fetus* subspecies - Cff, *Campylobacter fetus* subsp. *venerealis* (Cfv) and *Campylobacter fetus* subsp. *testudinum* (Cft), where the latter has been reported previous to grow aerobically. Data for reference taxa were defined based on previously reported species/subspecies descriptions (Véron and Chatelain 1973; Fitzgerald *et al.* 2014; Gilbert *et al.* 2017).

| | **Cff CITCf01** | **Cff CITCf02** | **Cff** | **Cfv** | **Cft** |
|---|---|---|---|---|---|
| Oxidase | + | + | + | + | + |
| Catalase | + | + | + | v | + |
| Urease | - | - | - | - | - |
| Nitrate reduction | + | + | + | v | + |
| Hippurate hydrolysis | - | - | - | - | - |
| Indoxyl acetate hydrolysis | - | - | - | - | - |
| $H_2S$ production | - | - | - | - | - |
| α-haemolysis | - | - | - | v | - |
| **Growth in/at/on** | | | | | |
| 18-22°C (microaerobic) | + | + | v | v | + |
| 25°C (microaerobic) | + | + | + | + | + |
| 37°C (microaerobic) | + | + | + | + | + |
| 42°C (microaerobic) | + | + | v | - | v |
| 37°C (anaerobic) | + | + | v | v | + |
| 37°C (aerobic) | + | + | - | - | v |
| CCDA | + | + | + | + | + |
| Glycine 1% | + | + | + | v | + |
| MacConkey agar | + | + | v | v | v |
| Nutrient agar | + | + | + | + | *nd* |
| **Resistance to** | | | | | |
| Nalidixic acid (30 µg) | + | + | + | v | + |
| Cephalothin (30 µg) | - | - | - | - | *nd* |

+, 90–100%; v, 11–89%; -, 0-10% of strains positive; *nd*, not determined

**Table 2.** Minimum inhibitory concentrations (MIC) and antimicrobial resistance of *Campylobacter fetus* subsp. *fetus* (Cff) isolates CITCf01 and CITCf02.

| Antimicrobial | Cff isolate CITCf01 | Cff isolate CITCf02 |
|---|---|---|
| | MIC (µg/mL) | MIC (µg/mL) |
| Tetracycline | ≤ 0.5 (S) | 1 (S) |
| Erythromycin | ≤ 1 (S) | ≤ 1 (S) |
| Ciprofloxacin | 0.5 (S) | 0.25 (S) |
| Gentamicin | 0.5 (S) | 0.5 (S) |
| Streptomycin | 2 (S) | 2 (S) |

S, sensitive; R, resistant

**Table 3.** Genomic attributes of *Campylobacter fetus* subsp. *fetus* isolates CITCf01 and CITCf02 and type strain NCTC 10842$^{T}$.

| | ***Campylobacter fetus* subsp. *fetus* isolate CITCf01** | ***Campylobacter fetus* subsp. *fetus* isolate CITCf02** | ***Campylobacter fetus* subsp. *fetus* type strain NCTC 10842$^{T}$ (presence/absence)** |
|---|---|---|---|
| Genome size (bp) | 1,781,331 | 1,769,856 | 1,763,253 |
| GC-content (%) | 33.3 | 33.3 | 33.3 |
| Protein coding sequences (# of ORFs) | 1,734 | 1,733 | 1,730 |
| tRNAs | 44 | 44 | 44 |
| rRNA | 9 | 9 | 9 |