**In silico** investigation of the genus *Campylobacter* type VI secretion system reveals genetic diversity in organization and putative effectors

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**Abstract**

Bacterial type VI secretion systems (T6SSs) are contractile nanomachines that deliver proteinic substrates into target prokaryotic or eukaryotic cells and the surrounding milieu. The genus *Campylobacter* encompasses 39 recognized species and 13 subspecies, with many belonging to a group known as ‘emerging Campylobacter pathogens’. Within *Campylobacter*, seven species have been identified to harbour a complete T6SS cluster but have yet to be comparatively assessed. In this study, using systematic bioinformatics approaches and the T6SS-positive *Campylobacter jejuni* 488 strain as a reference, we explored the genus-wide prevalence, similarity and make-up of the T6SS amongst 372 publicly available ‘complete’ *Campylobacter* genomes. Our analyses predict that approximately one-third of *Campylobacter* species possess a T6SS. We also putatively report the first identification of a T6SS in four species: *Campylobacter cuniculorum*, *Campylobacter helveticus*, *Campylobacter armoricus* and *Campylobacter ornithocola*. The *Campylobacter* T6SSs cluster into three distinct organizations (I–III), of which two break down into further variants. Thirty T6SS-containing genomes were found to be harbouring more than one vgrG gene, with *Campylobacter lari* strain NCTC 11845 possessing five. Analysis of the *C. jejuni* Pathogenicity Island-1 confirmed its conservation amongst T6SS-positive *C. jejuni* strains, as well as highlighting its diverse genetic composition, including additional putative effector–immunity pairs (e.g. PoNe and DUF1911 domains). Effector–immunity pairs were also observed neighbouring vgrGs in several other *Campylobacter* species, in addition to putative genes encoding nucleases, lysozymes, ATPases and a ferric ATP-binding cassette uptake system. These observations highlight the diverse genetic make-up of the T6SS within *Campylobacter* and provide further evidence of its role in pathogenesis.

**DATA SUMMARY**

All genomes used in this study were retrieved from the National Center for Biotechnology Information RefSeq genome database (www.ncbi.nlm.nih.gov/refseq) and metadata collected from the NCBI BioSample database (www.ncbi.nlm.nih.gov/biosample) (accession numbers and metadata available in Table S1, available in the online version of this article), except for *C. jejuni* strain 488 for which the raw fastq files were previously deposited in ENA under the study accession PRJEB41135, and the assembled gbk file can be found in our previous publication [1].

**INTRODUCTION**

Bacteria inhabit a multitude of environments where conflict engagement and resistance to stresses drive adaptation. To subsist in these hostile conditions, bacteria have established multiple physiological mechanisms to promote fitness. Bacterial secretion...
systems are a ubiquitous apparatus of protein transportation essential for interaction with the surroundings and neighbouring cells [2]. In gram-negative bacteria, they are categorized into Types I–X [2–4]. The type VI secretion system (T6SS) is a transmembrane proteinaceous nanomachine found in ~25% of Proteobacteria and contributes roles to interbacterial enmity, host–pathogen interaction, resistance to environmental stresses, natural transformation and endurance in competitive ecosystems [5–7]. Secretion of a panel of T6SS-specific effectors into target cells and the presence of cognate immunity proteins to prevent autotoxicity mediate these hostile interactions, ensuring kin protection and hindrance of competitors [5, 8]. Hence, effectors have been characterized to possess both antibacterial [9–11] and anti-eukaryotic activity [12].

Consisting of approximately 14 core components [13], the T6SS is abundant within gram-negative bacteria [14, 15]. Homologous features to the bacteriophage T4 tail-, baseplate- and membrane-like spanning structures assemble to form a bacterial contractile apparatus [16, 17]. Proteins TssJ, TssL and TssM interact to form a complex which anchors the secretion system to the membrane. The cytoplasmic baseplate (TssEFGK) of the apparatus then binds to the membrane complex, as does the contractile sheath (composed of TssB and TssC). A needle-like tube consisting of hexameric rings of the protein Hcp/TssD is then encased by the sheath and capped by the protein TssA at the opposing cellular membrane [13, 18]. The machinery also possesses a puncturing spike composed of trimers of the protein VgrG, a homologue of the phage spike proteins gp27/gp5 [17, 19]. This spike is further sharpened by a proline–alanine–alanine–arginine (PAAR)-repeat protein [20]. Upon contraction, the Hcp tube, VgrG-PAAR complex and any associated effectors are propelled across the bacterial membrane into adjacent target cells (with penetration facilitated by the spike) or the external environment [5]. Disassembly of the system is coordinated by the ATPase ClpV, a component that has not been identified to date in T6SS-harbouring Campylobacter species [18, 21–23]. Accessory proteins, such as the post-translational regulator Fha1 (TagH in Campylobacter jejuni), also contribute important roles to T6SS assembly and secretion of effectors [24–26]. Recognition of associated proteins can occur through the presence of motifs [20, 27], yet due to the lack of a widely distinguishable feature amongst secreted substrates, effectors are generally categorized into two classes: ‘cargo’ or ‘specialized’ [5]. The former represents effectors which covalently interact with one or two of the components of the cellular device before secretion [24, 28], whilst the latter exist as toxic domain-containing extensions of secreted structural components [25, 26].

The genus Campylobacter encompasses a collection of 44 proposed species, of which 39 names have been published and recognized by the International Code of Nomenclature of Prokaryotes, and 13 subspecies observed to colonize a range of environments [29–32]. This ability to ubiquitously inhabit diverse environmental conditions can frequently lead to infection in humans, known as campylobacteriosis [30]. Campylobacter is the leading cause of bacterial foodborne gastroenteritis worldwide, with C. jejuni as the causative agent of the majority of infections in humans and is therefore of significant clinical relevance [30, 33]. Several Campylobacter species belong to a group known as ‘emerging Campylobacter pathogens’, including Campylobacter concisus, Campylobacter fetus and Campylobacter lari, which opportunistically infect both animals and humans [29, 34, 35]. A small proportion of these documented Campylobacter species still possess only one available genomic sequence, such as Campylobacter ornithocola and Campylobacter rectus [29]. In comparison, C. jejuni has over 200 genomes of quality level ‘complete’ in the National Center for Biotechnology Information (NCBI) RefSeq database [36]. Within Campylobacter, several species are described to harbour a T6SS, with the earliest reported and predominantly investigated in C. jejuni [25, 36–40]. Studies have revealed the association of the C. jejuni T6SS to numerous physiological functions, including pathogenic interaction (colonization, invasion and adhesion), cytotoxicity and resistance to oxidative stress [25, 41]. Recently, we conducted a large comparative analysis of the T6SS in a population of publicly available C. jejuni genomes, determining the presence of a Campylobacter jejuni Integrated Element 3 (CJIE3)-variant termed Campylobacter jejuni Pathogenicity Island-1 (CJPI-1) harbouring the T6SS amongst a significant proportion of strains [1]. Other studies have also conducted similar taxonomic-scale analyses [38, 42], as well as briefly comparing the T6SS operon organization between species [25, 43]. Further, T6SS-harbouring genomic islands, integrative elements and plasmids have also been identified within several Campylobacter species [40, 44].
While several studies have called attention to the existence and importance of the T6SS in a growing number of pathogenic Campylobacter species, its (i) evolutionary origins, (ii) genus-wide prevalence, (iii) interspecific similarity, (iv) intraspecific genetic transfer, (v) range of associated effectors and (vi) contribution to pathogenesis remain largely unknown. We have therefore performed a bioinformatic analysis of 372 publicly available Campylobacter genomes encompassing 33 species and 17 unassigned strains, investigating the prevalence, genetic composition and make-up of the T6SS. The major T6SS components TssC, Hcp and VgrG, and a T6SS-harboring genetic element were investigated using the T6SS-positive C. jejuni 488 strain as a reference. A systematic prediction of genes neighbouring identified vgrGs was also conducted. We identified several potentially insightful inter- and intraspecific similarities and differences between T6SS-harboring genomes, thus expanding our current understanding of the T6SS diversity and roles.

**METHODS**

**In silico identification of T6SS-containing Campylobacter genomes**

Nucleotide and protein sequences of Campylobacter genomes were collected from the NCBI RefSeq genome database release 205 (March 2021) at assembly level ‘complete genome’ or higher [36]. The T6SS-positive C. jejuni 488 strain (a human isolate from Brazil) [1] was included in the genome dataset as a reference, and a local nucleotide and protein database was constructed from a total of 372 genomes. Metadata, including host and sample location, was collected from the NCBI BioSample database [45].

BLASTP (BLAST+ v2.12.0) [46, 47] was employed to identify the 13 T6SS components amongst the Campylobacter genomes, using an expected value (E-value) of 1e-10. Amino acid sequences of the 14 T6SS loci (two VgrGs) from the T6SS-positive C. jejuni 488 strain [1] were aligned against a local protein dataset created for the Campylobacter genomes. A similarity percentage was calculated by dividing the bit-score value for each amino acid alignment by twice the specific lengths of the individual query amino acid sequence to facilitate T6SS loci identification similarly to that reported previously [48]. Genome visualization was performed in Artemis (v14.0.12) to manually inspect the T6SS operon organization [49] and comparative visualization was conducted using Clinker (v0.0.23) [50], with default parameters. Domain prediction was conducted using NCBI CDD-BLAST [51], with default parameters.

**Phylogenetic analysis of all Campylobacter genomes under study**

To construct a phylogenetic tree of all Campylobacter genomes in the local dataset, BLASTP [46] was employed to identify and extract the protein AtpA from all genomes. AtpA (ATP synthase F1 complex alpha subunit) is used to sequence type C. jejuni and Campylobacter coli isolates by PubMLST [52] and is therefore commonly used to reconstruct phylogenetic trees of the genus Campylobacter [53, 54]. The protein sequence of CJ488_00072 (AtpA homologue) was first aligned against the local Campylobacter protein database. Then, extracted sequences were aligned using MUSCLE [55], with default parameters. A maximum-likelihood tree was then reconstructed with FastTree2 (v2.1.10) [56] using the alignment file and WAG+CAT model parameters. The tree was visualized using the Interactive Tree of Life (iTOL, v6) webtool (https://itol.embl.de) [57].

**Phylogenetic analysis of TssC and Hcp components**

BLASTP [46] was employed to identify and extract the components TssC and Hcp amongst the local Campylobacter genomes, using an E-value=1e-10. Protein sequences CJ488_00968 (Hcp) and CJ488_00974 (TssC) from C. jejuni 488 were aligned against the local Campylobacter protein database. Extracted sequences were then aligned using MUSCLE [55] with default parameters. A phylogenetic tree was reconstructed from the alignment file for each component using the maximum-likelihood method, with JTT modelling, partial deletion (95%) and bootstrapping (n=500) parameters, conducted in the Molecular Evolutionary Genetics Analysis X (MEGAx, v10.2.6) software package [58]. These analyses contained 62 amino acid sequences, respectively. Tree display was conducted using the iTOL (v6) webtool [57].

**Prediction of VgrG proteins harboured in Campylobacter genomes**

BLASTP [46] was employed to identify homologous VgrG proteins amongst the Campylobacter genomes, using an E-value=1e-10. The N-terminal amino acid sequence of VgrG1 and VgrG2 possesses >99% amino acid identity [1], and therefore only the N-terminal amino acid sequence of VgrG1, containing the VgrG domain (COG3501), from C. jejuni 488 was aligned against the local Campylobacter protein database to identify VgrG homologues. Sequences were then filtered to remove any of the traits: (i) sequence matched to a genome that was not considered to harbour a T6SS (e.g. C. jejuni CLB104), (ii) possessed a fragmented VgrG ORF (e.g. AEI23_RS09890 and AEI23_RS05295 from C. jejuni CJ018CCUA), (iii) internal stop codons present in the sequence (e.g. EL232_RS07845 from C. jejuni NCTC 13265), (iv) missing methionine start to the sequence, (v) less than 100 aa in length, and/or (vi) the VgrG domain was not identified using NCBI-CDD BLAST (e.g. AEI20_RS09140 from C. jejuni CJ071CC464). A total of 19 sequences were removed from further analysis. Domain prediction was conducted using NCBI-CDD [51] with default parameters.
In silico identification of CJPI-1-containing genomes

To identify the presence of CJPI-1 amongst the C. jejuni genomes from the local Campylobacter dataset, the CJPI-1 genes CJ488_0930 (CJPI-1 integrase), CJ488_0941 and CJ488_1004 from C. jejuni 488 were used in an in silico identification method as proxies [1]. CJ488_0941 and CJ488_1004 are homologues of C. jejuni RM1221 strain genes cje1105 and cje1153, respectively, harboured in the CJIIE3. blastn [46] was employed, using default parameters, to align the nucleotide sequence of CJ488_0930, CJ488_0941 and CJ488_1004 against the local nucleotide dataset created for the Campylobacter genomes. A similarity percentage was calculated according to Fридман et al. [48]. To be regarded as positive for CJPI-1, a minimum similarity of 50% was required for two of the three genes: CJ488_0930, CJ488_0941 and/or CJ488_1004. To be regarded as possessing a T6SS-harbouring plasmid, (1) a minimum similarity of 50% was required to just the gene CJ488_0930 and (2) the presence of at least 12 T6SS loci (T6SS-positive). Comparative genomic analysis of CJPI-1-harbouring C. jejuni genomes was conducted using the Artemis Comparison Tool (v18.1.0) [59]. Query cover was calculated using the blastn (megablast) webtool [46], with the nucleotide sequence of CJ488 acting as the query and the predicted CJPI-1 sequence in the other C. jejuni strain as the subject.

Functional prediction of genes downstream and upstream of vgrGs harboured by T6SS-positive Campylobacter genomes

Genes neighbouring vgrGs are commonly identified to possess toxic activity and are characterized as cognate effectors [24, 60]. Protein sequences for genes identified as one to five genes upstream (+5) or one to five genes downstream (−5) of vgrG genes identified in the previous analysis stage (94 sequences) were systematically extracted and collated. Amino acid sequences were then submitted to the NCBI CDD-blast (default parameters, concise results) [51], SignalP (v5.0, parameter – Gram-negative) [61] and TMHMM (v2.0, default parameters) [62] servers for functional prediction.

In silico identification of PAAR-like domain-containing sequences

blastp [46] was employed to identify PAAR-like domain-containing proteins [1] amongst the Campylobacter genomes, using an E-value=1e-10. The amino acid sequence of the PAAR-like domain (amino acids 25–150) from CJ488_00990 in C. jejuni 488 was aligned against the local Campylobacter protein database. Homologous sequences were then subject to domain prediction using NCBI CDD-blast and Pfam [51, 63], with default parameters.

Identified PAAR-like domain-containing protein sequences were then aligned using ClustalOmega [64] and visualized with WebLogo3 [65], with default parameters, to confirm the presence of conserved cysteine and histidine residues.

RESULTS

One-third of Campylobacter species in our local database harbour a T6SS

To estimate the prevalence of the T6SS amongst the genus Campylobacter, we first constructed a local database of Campylobacter genomes of assembly level ‘complete genome’ or higher (n=371) from the NCBI RefSeq database [36]. This was further populated with the T6SS-positive C. jejuni 488 strain as a reference, creating a total of 372 genomes (Table S1). Our local database consisted of 33 Campylobacter taxa and 17 strains unassigned to any known species. Genome size ranged from 1.465 Mbp (Campylobacter insulanaeigrae NCTC 12927) to 2.572 Mbp (C. rectus ATCC 33238). C. jejuni was the most abundant species in our database, contributing 212 genomes to the dataset, followed by C. coli (n=33) and C. fetus (n=20) (Fig. 1a). Metadata was also collected, of which the two most prevalent sources of isolation were humans (n=281) followed by avian species (n=34) (Fig. 1b).

Next, we screened the 13 core T6SS loci and orphan vgrG locus VgrG2 from T6SS-positive C. jejuni 488 against the locally constructed database, identifying 62 (16.66%) genomes to possess at least 12 of the 13 major T6SS components, and therefore classifying them as T6SS-positive (Tables S1 and S2). No T6SS-positive genomes were found to present in more than one copy of the complete T6SS cluster. Campylobacter curvum culicorum 2010D-8469 and C. curvum LMG 2588 were the only genomes predicted to harbour two tagH genes. C. jejuni FDAARGOS_421, NCTC13265, RM1221, RM1221 (2) and CLB104, C. coli AR-0411, and C. concisus P3UCB1 and P3UCO1 strains were identified to possess up to two of the screened T6SS components but were not considered to harbour a T6SS. In total, 11 species (33.33%) in our database were identified as possessing a T6SS operon, as well as a further five strains unassigned to a known species. Again, the most prevalent species was C. jejuni, with 23 strains predicted to harbour a T6SS, followed by C. concisus (n=14) and C. coli (n=7) (Fig. 1c). Phylogenetic analysis of all Campylobacter genomes under study further highlighted the spread of the T6SSs within the genus, with distribution across the genus rather than within distinct groups (Fig. 2).

Of the T6SS-positive genomes, 46 (74.19%) were isolated from humans as hosts, six (9.68%) from avian species, and ten (16.13%) from other or unknown species (defined as all species not considered human or avian in the metadata) and environmental isolates. Therefore, among the genomes under study, 16.37% were identified as T6SS-positive within all human isolates (n=281), 17.65% within all avian species isolates (n=34), and 17.54% within all other species or unknown isolates (n=57). Among the 11 species observed to encode a T6SS, to the best of our knowledge, we putatively report the first identification of a T6SS in four
species: *C. cuniculorum*, *Campylobacter helveticus*, *Campylobacter armoricus* and *C. ornithocola*. *C. armoricus* CA639, *C. helveticus* 2013D-9613 and ATCC 51209, and *C. ornithocola* LMG 29815 were predicted to harbour all 13 T6SS components (*tssA–tssM, hcp, vgrG* and *tagH*), whilst both *C. cuniculorum* strains, mentioned previously, harbour all 13 and an additional putative *tagH* gene. Both encoded *tagH* genes in the *C. cuniculorum* strains possessed 99.67% nucleotide sequence identity when compared to each other.

**T6SS-positive Campylobacter species consist of three T6SS cluster organizations**

After determining the prevalence of the T6SS, we next investigated the organization of the T6SS clusters amongst the T6SS-positive genomes. During a systematic assessment of the clusters, we putatively predict three T6SS organizations (I–III) (Fig. 3a), of which two break down into further variants. T6SS organizations I-a to I-d possess a core set of nine components (*tssL to tssG*) which cluster together in the same orientation. Observable differences between the variants are determined by the location of the gene *vgrG* and *tagH-tssM-hcp* genetic cluster. In both I-a and I-b, the genes *tagH-tssM-hcp* are localized at the N-terminus of the main cluster adjacent to *tssL*, whilst the location of the *vgrG* varies. Alternatively, in organizations I-c and I-d, multiple genetic loci separate the main cluster from the genes *tagH-tssM-hcp*, and in the case of I-d, several *vgrGs* and effectors (see below) are harboured within this genetic region.

T6SS II possesses a unique organization that differs significantly from the T6SS I:

1. Genes *tssl-tssK-tssl* are encoded in the reverse orientation to their homologues in the T6SS I and III organizations. As a result, all genes of the cluster are encoded in the same transcriptional direction and suggest a coregulated expression.
2. Genes *tssM-tagH-hcp* are found at the C-terminus of the main cluster adjacent to the gene *tssG*.
3. Regions of genetic variation are found between genes *hcp* and *vgrG*.

The final organizations T6SS III-a and III-b were unique to one strain in the database, respectively. The organizations of III-a and III-b both possessed genes *tssM-tagH* at the C-terminus of the main cluster, similar to T6SS II, but the orientation of *tssl-tssK-tssl* was similar to T6SS I. Observable differences between variants III-a and III-b lie with the location of the genes *hcp* and *vgrG*. In T6SS organization III-a, the genes *hcp-vgrG* are found distant from the C-terminus of the main cluster separated by more than 10 genes, whereas in organization III-b both *hcp* and *vgrG* are encoded at the N-terminus with several genes separating *hcp* and *vgrG*. 

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**Fig. 1.** Dataset characteristics for the analysed *Campylobacter* species under study. (a) Pie chart showing the distribution of *Campylobacter* species within our local database. The number of isolates per species in our dataset is given next to the species name. (b) Bar graph showing the percentage distribution of isolation source for each *Campylobacter* species within our local database. (c) Bar graph showing the prevalence of T6SS-positive strains within the *Campylobacter* species in our dataset.
All *C. jejuni* strains were identified to possess the T6SS organization I-a, as well as *Campylobacter* sp. CFSAN093227, CFSAN093246, *C. cuniculorum* 2010D-8469 and LMG 2588, and all *C. coli* strains, except for strain ZV1224 which exclusively possessed organization I-b (Table S1). Organization I-c was observed in the genomes of *C. helveticus* 2013D-9613 and ATCC 51209. Genomes of strains *C. armoricus* CA639, *C. ornithocola* LMG 29815, *C. lari* RM16712, *Campylobacter* sp. 2014 D-0216, *Campylobacter peloridis* 2016D-0074 and LMG 23910, *Campylobacter subantarcticus* LMG 24374 and LMG 24377, and *C. lari* NCTC 11845 were identified to harbour the T6SS organization I-d. The number of vgrG genes carried by the genomes with T6SS organization I-d varied from one to five within the genetically variable region. All *C. concisus* genomes were classified to present the T6SS II organization (Fig. 2), although strain P26UCO-S2 was identified to lack the gene *tssJ* and strain P27CDO-S2 harboured a split *tssG* gene. Organizations T6SS III-a and III-b were solely observed in *C. rectus* ATCC 33238 and *Campylobacter* sp. CCUG57310, respectively (Table S1). Analysis of the core component TssC was also performed between the three T6SS organizations and their variants, confirming higher homology within the distinct T6SS organizations than to each other, suggesting the organization variants are related (Fig. 3b).

**Interspecies phylogenetic analysis of T6SS-positive *Campylobacter* species**

Deletion of *tssC* in the *C. jejuni* 488 strain leads to a reduction in TssD (Hcp) secretion and thus is an important constituent of T6SS functionality [23]. Component Hcp has also been suggested as a putative effector in *C. jejuni* and is therefore likely to present species-specific traits to prevent immunity during T6SS-mediated conflict [37, 66]. We, therefore, chose to infer a phylogeny...
for genomes harbouring a T6SS under study (n=62) by producing two maximum-likelihood phylogenetic trees based on the core-sheath component TssC and secreted component Hcp as they each appear to contribute distinct roles to the activity of the *Campylobacter* T6SS. Both trees produced similar topological arrangements of T6SS-positive species and strains (Fig. 4a and b). We observed an overall high diversity within strains as several small subgroups could be identified, with clustering dependent on the T6SS cluster organization (Fig. 4a). Grouping for the Hcp phylogenetic tree also followed a similar topology but could

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**Fig. 3.** The three distinct T6SS organizations in the genus *Campylobacter*. (a) Comparative visualization of the genus *Campylobacter* T6SS organizations highlighting the variable nature of the operon. Genes coloured the same with connected bands are homologues. Genes found in only one genome possess a grey arrow. Arrowheads represent gene direction for transcription. Corresponding locus tags or gene names are given above the respective ORFs. The figure was generated using Clinker [50]. (b) Amino acid identity of the TssC homologues from the three T6SS organizations and their variants to each other. Amino acid sequence identity was calculated with BLAST Global Alignment–Protein (Needleman–Wunsch Global Align) [46]. The *C. jejuni* 488 strain is used as a representative genome for the T6SS I-a organization, *C. coli* ZV1224 for the I-b organization, *C. helveticus* 2013D-9613 for the I-c organization, *C. peloridis* 2016D-0074 for the I-d organization, *C. concisus* H101 for the II organization, *C. rectus* ATCC 33238 for the III-a organization and *C. sp.* CCUG 57310 for the III-b organization.

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**Fig. 4.** Phylogenetic trees derived from a maximum-likelihood analysis on MUSCLE alignments of (a) TssC and (b) Hcp amino acid sequences from T6SS-positive *Campylobacter* species (bootstrap n=500, partial deletion) and visualized using iTOL [57]. The scale bar indicates the genetic distance, and the bootstrap value of each node is given. The node colour indicates T6SS organization: red=I-a, yellow=I-b, green=I-c, teal=I-d, orange=II, blue=III-a and burgundy=III-b.
be further clustered into two larger clades, potentially due to the absence of outliers (Fig. 4b), as observed by the sequence for TssC from *C. jejuni* YQ2210 in Fig. 4a.

**Approximately half of T6SS-positive Campylobacter species investigated harbour multiple VgrGs**

Recently, we identified the presence of two distinct vgrGs harboured by the T6SS-positive strain *C. jejuni* 488 [1]. To assess the prospect of multiple vgrGs harboured by other T6SS-positive *Campylobacter* species, we extracted the amino acid sequences from our local protein database that matched the VgrG domain (COG3501, amino acids 45–563) of VgrG1 (CJ488_00978) from *C. jejuni* 488. In total, 113 homologous sequences were identified initially, of which 19 were subsequently removed due to criteria described in the Methods. The remaining 94 sequences were subject to further analysis. The average number of vgrG sequences identified amongst the T6SS-positive genomes was greater than one (*n* = 1.633), with 30 (48.39%) genomes harbouring more than one VgrG (Figure 1). Notably, five vgrGs were identified in *C. lari* NCTC 11845, located in the genetic region between genes *hcp* and *tssL* of T6SS organization I-d. Lengths of VgrGs also varied greatly, with *C. concisus* H9O-S2 harbouring a VgrG of 1025 aa, whilst the shortest of 430 aa was identified in multiple strains. VgrG sequences were then analysed to identify conserved and additional domains. As expected, all sequences across all strains were found to harbour the conserved domains VgrG (COG3501) and VI_Rhs_Vgr superfamily (cl37255) in the N-terminus (Table S3). Several strains were predicted to harbour the _5_superfamily (cl33691) domain previously observed in the sequence of VgrG2 from *C. jejuni* YQ2210 in Fig. 4a. Notably, five *C. lari* NCTC 11845, located in the genetic region between genes *hcp* and *tssL* of T6SS organization I-d. Lengths of VgrGs also varied greatly, with *C. concisus* H9O-S2 harbouring a VgrG of 1025 aa, whilst the shortest of 430 aa was identified in multiple strains. VgrG sequences were then analysed to identify conserved and additional domains. As expected, all sequences across all strains were found to harbour the conserved domains VgrG (COG3501) and VI_Rhs_Vgr superfamily (cl37255) in the N-terminus (Table S3). Several strains were predicted to harbour the _5_superfamily (cl33691) domain previously observed in the sequence of VgrG2 from *C. jejuni* 488 [1]. Several other domains were also observed within the VgrG sequences but considered non-significant (Table S3).

**CJPI-1 is a diverse and mobile genetic island**

Following on from our previous study [1], we next investigated the prevalence and diversity of the ‘CJIE3-variant’ CJPI-1 amongst the *C. jejuni* strains under study. *In silico* identification and visualization in Artemis [49] was then used to confirm the genomic location of the T6SS (i.e. genomic island or plasmid). All T6SS-positive *C. jejuni* strains (n = 23) in our dataset harboured a T6SS either within the CJPI-1 (n = 18) or on a plasmid (n = 5) (Tables S1 and 4). Comparative analysis of the newly identified CJPI-1 amongst *C. jejuni* strains against CJPI-1 from *C. jejuni* strain 488 revealed considerable diversity in length and genetic composition between harbouring strains (Table 1); however, chromosomal incorporation was consistently adjacent to a tRNA and a query coverage of greater than 75% was observed. In comparison, the CJIE3 from T6SS-negative *C. jejuni* RM1221 possesses a query cover of 45% when aligned against CJPI-1 from T6SS-positive strain 488, suggesting closer genetic similarities between CJPI-1s from T6SS-positive *C. jejuni* strains than to CJIE3. Assemblies of *C. jejuni* genomes CJ018CCUA and CJ017CC64 were removed from the comparative analysis due to potential *in silico* errors. We assume minor inaccuracies in the assemblies as genes that were predicted within the putative CJPI-1 and other genomic loci were found dispersed towards the end of the assembly file. Consequently, we could not accurately predict the length, G+C content and/or position of the CJPI-1 during the comparison to strain 488.

Twelve (5.63%) out of 212 *C. jejuni* genomes were also identified to possess the integrative element CJIE3 (Table S1). Additionally, several non- *C. jejuni* species were putatively identified to possess synonymous T6SS-harbouring islands, as well as T6SS-encoding plasmids observed in *Campylobacter* sp. CFSAN093246 and *C. coli* strains 14983A, FDAARGOS_1028, meC0281, YH502 and YH503.

**T6SS-positive Campylobacter species encode multiple putative effector subsets neighbouring VgrGs**

Recently identified within CJPI-1 of *C. jejuni* 488, four putative effectors and two immunity genes were predicted to be encoded within a genetically variable region between the genes vgrG1 and vgrG2, representing the first potential secreted substrates to be utilized by the T6SS in *Campylobacter* [1]. We hypothesized that other effectors and secreted proteins could be found neighbouring vgrGs in the previously identified T6SS-harbouring *Campylobacter* species. To achieve this, we systematically extracted the protein sequences of the five upstream (+5) and downstream (−5) genes adjacent to identified vgrG homologues across all T6SS-positive species under study. Extracted sequences were then subject to functional characterization through domain, signal peptide and transmembrane helix analyses (Table S5).

We predicted two effector–immunity pairs amongst the T6SS-harbouring population. The first pair consisted of proteins harbouring domains belonging to the Ankyrin (PF12796/cl33707) and Tox-REase-7 (cl21441) superfamilies (e.g. A6J90_RS09495 and A6J90_RS06400 in *C. jejuni* M129) and was identified upstream of the cognate vgrG (Figure S1). Notably, five *C. lari* NCTC 11845, located in the genetic region between genes *hcp* and *tssL* of T6SS organization I-d. Lengths of VgrGs also varied greatly, with *C. concisus* H9O-S2 harbouring a VgrG of 1025 aa, whilst the shortest of 430 aa was identified in multiple strains. VgrG sequences were then analysed to identify conserved and additional domains. As expected, all sequences across all strains were found to harbour the conserved domains VgrG (COG3501) and VI_Rhs_Vgr superfamily (cl37255) in the N-terminus (Table S3). Several strains were predicted to harbour the _5_superfamily (cl33691) domain previously observed in the sequence of VgrG2 from *C. jejuni* 488 [1]. Several other domains were also observed within the VgrG sequences but considered non-significant (Table S3).
Table 1. Analysis of CJPI-1s found in *C. jejuni* strains under study.

Data were collected using the Artemis comparison tool (v18.1.0) [49] and BLAST webtool [46].

| *C. jejuni* strain ID | Length of CJPI-1 (kb) | Chromosome position (nt) | No. of genes | G+C content (%) | Genome G+C content (%) | Integrase locus | Query coverage (%) |
|-----------------------|------------------------|--------------------------|--------------|-----------------|------------------------|----------------|-------------------|
| 488                   | ~70.3                  | 883538–953903            | 77           | 26.73           | 30.26                  | CJ488_0930 c   | 100               |
| 0-1597                | ~61.4                  | 941969–1003354           | 51           | 26.63           | 30.37                  | PJ17_RS04950   | 75                |
| 14980A                | ~103.5                 | 913056–1016609           | 95           | 26.65           | 30.25                  | CJ14980A_RS04755 | 86               |
| AR-0414               | ~74.9                  | 827033–901979            | 68           | 26.95           | 30.36                  | F4V26_RS04610  | 96                |
| CFSAN054107           | ~104.7                 | 1437320–1542021          | 98           | 26.45           | 30.15                  | C9J79_RS07600  | 93                |
| CJ018CCUA             | NA                     | NA                       | NA           | NA              | NA                     | NA             | NA                |
| CJ071CC464            | NA                     | NA                       | NA           | NA              | NA                     | NA             | NA                |
| FDAARGOS_262          | ~117.8                 | 1107844–1225628          | 117          | 26.44           | 30.22                  | A690_05940     | 91                |
| IF1100                | ~61.7                  | 893701–955418            | 61           | 26.80           | 30.23                  | BLD34_RS04785  | 79                |
| LDG17f                | ~92.3                  | 975284–1067564           | 76           | 26.78           | 30.30                  | E8N02_RS05125  | 97                |
| M129                  | ~79.5                  | 936529–1016065           | 74           | 26.95           | 30.29                  | CJM129_RS04930 | 96                |
| MTVDSCj16             | ~85.7                  | 1010779–1096499          | 94           | 26.46           | 30.29                  | MTVDSCj16_RS05475 | 81               |
| NCTC11351             | ~115.8                 | 940199–1056036           | 101          | 26.43           | 30.23                  | AT682_RS04900  | 90                |
| NCTC13268             | ~92.0                  | 950800–1042778           | 89           | 26.52           | 30.26                  | EL228_RS04925  | 92                |
| TS1218                | ~88.9                  | 950413–1039331           | 84           | 26.65           | 30.25                  | BLD42_RS05120  | 87                |
| YH803                 | ~71.0                  | 974153–1045144           | 63           | 26.93           | 30.35                  | FNK00_RS05145  | 95                |
| YQ2210                | ~92.9                  | 930122–1023004           | 90           | 26.67           | 30.13                  | BLD41_RS05290  | 90                |
| ZP3204                | ~91.9                  | 940911–1032787           | 94           | 26.58           | 30.23                  | BLD40_RS05340  | 90                |
| **Average**           | **88.93**              | **83**                   | **83**       | **26.67**       | **30.26**              | **NA**         | **89.88**         |

NA: Not applicable.
domain-containing proteins encoded by +5 or −5 genes were observed in the genomes of 10 *C. jejuni* strains, five *C. coli* strains, *C. sp. CFSAN093227* and *C. sp. CFSAN093238*, and *C. lari* NCTC11845 and RM16712. Lysozyme/endolysin domain-containing proteins were predicted in the genomes of four *C. concisus* strains and *C. sp. CCUG 57310*. Interestingly, *C. sp. CCUG 57310* was also predicted to encode a lysozyme immunity (cl34764) domain-containing protein, CORI_RS01180, adjacently upstream of its lysozyme (cl00222) domain-containing protein, CORI_RS01185. Additionally, five putative ATPase (cl34796) encoding proteins were also predicted near *vgrGs* in the genomes of *C. peloridis* 2016D-0074 and LMG 23910 and *C. lari* NCTC11845, as well as putative ATP-binding cassette (ABC) Fe³⁺-siderophore transport system (cl00262, COG1120, PF01032 and cl36974/COG4771) domain-containing proteins in *C. concisus* P27CDO-S2 (Fig. 5), and FepB protein encoding genes in four other *C. concisus* strains (Table S5). 

Of note, the most frequently observed proteins that encoded identifiable domains (n=89) either −1 or +1 genes neighbouring *vgrGs* were predicted as Ankyrin domain-containing (37.08%, n=33), TssG (34.83%, n=31) or Hcp (11.23%, n=10) proteins. Similarly, predicted proteins encoded by either +2 or −2 genes with identifiable domains (n=85) were predicted as TssF (36.74%, n=31), Tox-REase-7 (31.76%, n=27), TssM (10.59%, n=9) or Lysozyme/Endolysin (5.88%, n=5) proteins (Table S5).

**A PAAR-like domain is widespread amongst T6SS-harbouring Campylobacter species**

A bioinformatic screening for the PAAR-like domain identified in the protein CJ488_0990 (amino acids 25–150) from *C. jejuni* 488 [1] was also conducted against the local *Campylobacter* database to assess its prevalence. As a result, 240 homologous protein sequences were predicted to harbour the domain across the *Campylobacter* genomes, including both T6SS-harbouring and T6SS-absent genomes (Table S6). This was further confirmed by aligning the homologous sequences and visualizing the presence of the conserved cysteine and histidine residues (Fig. S2). The PAAR-like domain-containing sequences were then analysed for additional domains using NCBI-CDD and Pfam [51, 63]. Several proteins were subsequently found to harbour additional toxic domains. The toxin domain TNT (PF14021, n=14) and PoNe (cl41756, n=19) superfamily were consistently found in the C-terminus of select PAAR-like domain-containing proteins (Fig. 5). Further, several PAAR-like domain-containing proteins harboured a lipase (cd00519, n=19) superfamily in their N-terminus.

PAAR-like domain-containing proteins identified in the previous stage of analysis were also cross-referenced with the genes extracted from +5 upstream to −5 downstream of the *vgrGs*. Sixteen proteins encoded by genes neighbouring *vgrGs* were predicted to contain the PAAR-like domain, of which nine (56.25%) also possessed C-terminal PoNe toxin domains. The PAAR-like domain-containing proteins encoded by genes +5 to −5 of the identified *vgrGs* were found in the genomes of *C. jejuni* 14 980A, FDAARGOS_262, NCTC11351 and NCTC13268, *C. peloridis* 2016D-0074 and LMG 23910, *C. lari* NCTC11845 and RM16712, and *C. subantarcticus* LMG 24377 (Table S5).

**DISCUSSION**

The T6SS is a multifunctional weapon with a broad range of targets due to its array of deployable toxic effectors [12, 69]. The function of the *Campylobacter* T6SS has been explored in *C. jejuni*, contributing to different physiological mechanisms to promote...
pathogenesis [25, 41]. In this study, we identified within a subset of publicly available Campylobacter genomes that 33% of Campylobacter species under study putatively possess a T6SS. We note that Campylobacter ureolyticus DSMZ 20703 was previously reported to encode homologues of T6SS components [40], but it was not present in the assembly data downloaded from NCBI and thus not investigated. We also note that several recently identified novel species of the genus Campylobacter were also absent from the NCBI RefSeq release 205 database, including Campylobacter aviculæ, Campylobacter estrellidurum, Campylobacter taenioopiæ [70], Campylobacter portucaleñsis [71], Campylobacter novaezælandiæ [72], Campylobacter massiliensis [73] and Campylobacter vulpis [74], of which two were discovered after the database was downloaded. As such, our database does not encompass all Campylobacter species to date, and therefore we stress that the results of our study are exclusive to species only included in the local database.

It is also important to state that although many T6SS-harbouring species included in this analysis possess only one sequenced isolate, we cannot consider the genomes to be representative of the species. Variation in intraspecific T6SS prevalence within Campylobacter species is evident [1, 75, 76]; therefore, we recommend not to consider these isolates as ‘representative’ of the species but included them only to incite further exploration into the presence of the T6SS amongst Campylobacter species. Moreover, the reverse can also be stated for the absence of a T6SS in select species. Species and strains other than those included in our ‘complete’ genome dataset may possess a T6SS, such as the recently identified Campylobacter species mentioned above, warranting further investigation. Unsurprisingly, our study demonstrates the presence of the T6SS within Campylobacter is not solely restricted to one or two species, similarly to other T6SS-harbouring genera [15, 77]. Its widespread presence has probably been achieved by events of genetic acquisition [78, 79]. Ultimately, this would independently lead to the same beneficial traits for distinct survival motives, such as competition or niche survival [80]. Since 2012, seven Campylobacter species have been identified to encode a complete T6SS [37–40, 43]. Here, we add a further four with the prediction of a T6SS in C. cuniculorum, C. helveticus, C. armoricus and C. ornithocola. We hope that reporting the presence of a T6SS in these species advances the knowledge about their putative virulence, in addition to developing strategies to control their distribution and pathogenicity. We also anticipate this novel finding to be the ‘tip of the iceberg’ for the presence of a T6SS in these species advances the knowledge about their putative virulence, in addition to developing strategies to control their distribution and pathogenicity. We also anticipate this novel finding to be the ‘tip of the iceberg’ for the presence of a T6SS in these species advances the knowledge about their putative virulence, in addition to developing strategies to control their distribution and pathogenicity.

Prevalence of the T6SS in C. jejuni isolates from humans has been observed to vary between low- and middle-income and high-income countries [76]. In this study, T6SS-harbouring isolates were observed predominantly with humans as the source of isolation. However, it is important to note that this observed association is probably due to the partiality to sample and upload of genomes of clinically relevant isolates, such as those from humans, rather than environmental isolates; therefore, over-representation of their prevalence in our dataset is likely. This is also in addition to potential sequencing bias of isolates from other non-human species prohibiting the inference of T6SS prevalence in different hosts. Further research using isolates from singular hosts is needed to explore the associations between the Campylobacter T6SS and humans, as well as disease severity.

Numerous T6SS-harbouring genera possess several T6SSs within their genomes clustered into distinct organizations [82, 83]. Here, we predict three distinct genetic organizations encoding the components for a singular T6SS cluster within Campylobacter. Discrepancies between the T6SS variant organizations of I and III imply genetic reshuffling, potentially arbitrated by horizontal gene transfer, has allowed translocation of the vgrG gene. This may have occurred through its possession of a ‘rearrangement hotspot’ element, previously observed in C. jejuni [22]. Phylogenetic analysis of all Campylobacter genomes under study and T6SS-positive isolates also highlighted the conservation of T6SS cluster organizations within distinct species. T6SS organization II and variants of organization III were exclusively found in one species, respectively, whereas T6SS organization I was identified in 10 species (Figs. 2 and 4). To deduce why particular organizations are favoured by specific Campylobacter species is beyond the scope of this study, but factors such as the source of isolation (i.e. host), genomic location (e.g. chromosome, plasmid), effector utilization and conservation of energy may contribute to their determination. T6SS organization II potentially demonstrates this prospective benefit of energy conservation by encoding its T6SS genes in the same transcriptional direction, thus relying on a singular promoter to co-regulate the expression of the entire T6SS cluster as one operon. Furthermore, as observed in this study and our previous work, T6SS cluster-containing mobile genetic elements could also lead to the dissemination of organizations among species with homologous recombination potentially facilitating chromosomal integration [1].

Encoding multiple T6SS clusters also leads to the presence of several structural component homologues across genomes. T6SS-harbouring species can encode multiple vgrGs and hcps distributed between main and orphan clusters, exhibiting diverse functions [12, 19, 67, 84]. Two distinct vgrG genes were putatively identified by our group in the CJPI-1 of the C. jejuni 488 strain, with homologues to both in C. coli genomes [1]. Although all T6SS-harbouring species were observed to possess one T6SS cluster, our prevalence study identified a significant proportion of the T6SS-harbouring species encoding two or more vgrGs, as well as a conserved domain across all VgrG protein sequences. All additional vgrGs were found distant from the main cluster. We speculate that harbouring diverse VgrGs within the genus Campylobacter provides unique advantages...
to encoding species and strains, particularly related to the diverse environments they inhabit. Typically, interchange of the spike complex VgrG trimer leads to the specific interaction of effectors via the protein’s C-termini, widening the range of cellular targets [12].

Pathogenicity islands are a unique subset of mobile genetic elements, conferring specific virulence phenotypes to harbouring bacteria governed by the genes carried [85]. In this study, we sought to verify the prevalence of CJPI-1 amongst our identified T6SS-positive C. jejuni population, in conjunction with exploring its prospective variation and make-up. Through comparative approaches, we found that 18 T6SS-harbouring C. jejuni strains encoded their T6SS within the CJPI-1, with the remainder harbouring theirs on discrete plasmids. We note considerable variation in the length and genetic composition of CJPI-1 between the strains under study, suggesting multiple events of genetic exchange have resulted in the acquisition or loss of specific genes. A select few were observed to possess additional toxin–antitoxin (TA) modules (data not shown) not detected in our previous study, whilst others harboured supplemental effector–immunity pairs in the downstream genetically variable region [1]. TA modules are prominent modulators of mobile genetic element persistence [86]; accordingly, encoding several TA modules within CJPI-1 suggests an important role. Either CJPI-1 is a significant constituent for the survival of harbouring strains and the cognate TA modules are responsible for its stabilization and fitness, or the TA modules are accumulated for other functions, namely utilization by the T6SS. Interestingly, we also noted several other Campylobacter species to harbour mobile genetic elements and plasmids that code for T6Sss, meriting further investigation.

Genes neighbouring vgrGs are frequently identified as secreted substrates for the cognate spike proteins, presenting a strategic investigative route to uncover associated toxic effectors [28, 67]. After systematically extracting the upstream and downstream protein sequences of genes adjacent to the Campylobacter vgrGs identified, we observed several putative effector and immunity proteins. Predicted effectors encoded nuclease domains Tox-REase-7, Tox-AHH and PoNe, as well as lysozyme/endolysin and lipase domains. These nuclease domains have been previously identified in several polymorphic protein delivery systems, including the T6SS [67, 68]. Immunity proteins were then confidently predicted adjacent to two of the effector-encoding genes, Tox-REase-7 and PoNe, with the PoNe-DUF1911 pair expanding upon the current repertoire of predicted effectors present within C. jejuni strains [1].

In addition to the predicted effectors, we also identified a putative ABC Fe³⁺-siderophore import system immediately downstream of a cognate vgrG (CVT17_RS00555 – CVT17_RS00570) in C. concisus strain P27COD-S2 (Fig. 5). Investigation into the domains harboured by the proteins predicted the existence of a putative FepBCD ferrienterobactin ABC transporter system, commonly found in Escherichia coli, and a CirA (COG1629)/FepA (COG4771) ligand-gated outer membrane receptor protein [87, 88]. To date, several secreted metal scavenging proteins are described for the T6SS, including TseF in P. aeruginosa which facilities iron acquisition [89, 90]. Iron is an abundant and essential element involved in both metabolic and cellular processes. As such, insoluble ferric iron (Fe³⁺) acquisition is a vital component of iron homeostasis in bacteria [91]. During periods of iron deficiency or competition for resources, Fe³⁺ acquisition strategies commonly involve the secretion of ferric-chelating siderophores and internal translocation via receptor uptake systems [92, 93]. Several ferric uptake systems are reported in Campylobacter, such as the CirA/CeuBCDE system in C. jejuni [94]. To the best of our knowledge, this is the first report of a putative FepABCD uptake system in the genus Campylobacter. Periplasmic binding protein FepB was also found downstream of vgrGs in four other C. concisus strains under study, but the permease, ATPase and outer membrane receptor were not. It remains unknown how C. concisus utilizes this ferric uptake system, as well as which ferric-siderophore is responsible for environmental Fe³⁺ acquisition. One hypothesis is that the C. concisus FepABCD system is scavenging siderophores produced by other competing bacteria. On the other hand, it is also possible that a distant chromosomal gene to the vgrG is encoding the metal scavenging siderophore, potentially secreted by the cognate secretion system; however, it is important to note that the FepABCD system is encoded in the opposing transcriptional orientation to the vgrG.

PAAR domains are encoded in several bacterial pathogens, frequently harboured in the N-terminus of proteins with C-terminal toxin domains [20, 95]. A PAAR-like domain-containing protein encoded in the CJPI-1 of T6SS-positive C. jejuni 488 [1] was screened against our local Campylobacter database to investigate its distribution and genetic context. We identified the presence of the PAAR-like domain across several species and strains within the genus Campylobacter, with a subdivision encoding toxic domains in their C-termini and others harbouring lipase domains at their N-termini. The existence of this domain across T6SS-positive species indicates a shared function of PAAR-like-containing proteins, further supported by the presence of conserved cysteine and histidine residues. A PolC (cl35100) superfamily domain, associated with the gram-positive bacterial DNA polymerase III gene polC [96], was also identified in 53 sequences presenting the PAAR-like domain; however, cross-checking in Pfam, due to the uncharacteristic function of the domain in conjunction with the role of PAAR and weak E-value, did not identify the domain using the predictive program. For this reason, we infer that the presence of this domain amongst PAAR-like domain-containing proteins is unlikely but warrants further investigation. We predict that the Campylobacter PAAR-like domain-containing protein is fulfilling both the role of sharpening the spike complex and simultaneously facilitating the transport of toxin domains to the secretion apparatus [20].
CONCLUSION

In summary, this study provides the most in-depth analysis of T6SS prevalence and diversity within the genus Campylobacter to date. We demonstrate genetic similarities and differences between the T6SS of Campylobacter species, as well as variation in the number of vgrGs, PAAR-like proteins and putative effectors harbouring by T6SS-containing genomes. We also report the putative first identification of a T6SS in four Campylobacter species, with other T6SS-harbouring species belonging to the growing group of ‘emerging Campylobacter pathogens.’ Understanding the arsenal of effectors this secretion system utilizes and the mechanisms by which the apparatus can be genetically transferred will provide a foundation for future experiments characterizing the Campylobacter T6SS.

Funding information
This work received no specific grant from any funding agency.

Author contribution
L.R. conceptualized the study. O.G. supervised the study. L.R. performed all data collection analysis and investigation. L.R. and O.G. interpreted the data. L.R. and O.G. contributed to the conception, writing, reviewing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

Conflicts of interest
The author(s) declare that there are no conflicts of interest.

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