Genomic analysis of the diversity, antimicrobial resistance and virulence potential of *Campylobacter jejuni* and *Campylobacter coli* strains from a private health care center in Central Chile

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ABSTRACT

Campylobacter jejuni and *Campylobacter coli* are the leading causes of human gastroenteritis in the developed world and an emerging threat in developing countries. There is increasing evidence that the incidence of campylobacteriosis in Chile and other South American countries is greatly underestimated, mostly due to the lack of adequate diagnostic methods. Accordingly, there is limited genomic and epidemiological data from these countries, leaving an important knowledge gap in our understanding of the population structure, virulence potential, and antimicrobial resistance profiles of clinical *Campylobacter* strains. We recently published the whole-genome sequences of a collection of 68 *C. jejuni* and 12 *C. coli* strains isolated from patients with enteric infections during a 2-year period (2017-2019) attending Clinica Alemana in Santiago, Chile. In the present study, we sequenced an additional *C. jejuni* genome and performed a genome-wide analysis of the genetic diversity, virulence, and antimicrobial resistance potential of these 81 strains. cgMLST analysis identified a high genetic diversity of *C. jejuni* as well as 13 novel STs and alleles in both *C. jejuni* and *C. coli*. Pangenome and virulome analyses showed a differential distribution of virulence factors, including both plasmid and chromosomally encoded T6SSs and T4SSs. Resistome analysis predicted widespread resistance to fluoroquinolones, but a low rates of erythromycin resistance. This study provides valuable genomic and epidemiological data and highlights the need for larger genomic epidemiology studies to better understand the genetic diversity, virulence potential and antimicrobial resistance of human clinical *Campylobacter* strains in South America.

DATA SUMMARY

1. Genome sequences and assemblies of all *C. jejuni* and *C. coli* used in this study have been deposited in public databases (**Table S1**) and most strains have also been published as genome resource announcements [1,2].

2. Bacterial draft genome sequence reannotations and nucleotide sequence fasta files used for virulome and plasmid screening analysis are available as a supplementary dataset on Zenodo (DOI: 10.5281/zenodo.3925206) https://doi.org/10.5281/zenodo.3925206.

3. The reconstructed phylogenetic trees and associated metadata of clinical strains from Chile in the global context of *C. jejuni* and *C. coli* sequenced strains can be interactively visualized on Microreact at https://microreact.org/project/VbEQsZtQD.

INTRODUCTION

Campylobacteriosis caused by *Campylobacter jejuni* and *Campylobacter coli* has emerged as an important public health problem and is the most common bacterial cause of human gastroenteritis worldwide [3–5]. Although the mortality rates associated with campylobacteriosis are low (<0.01% to 8.8%), this foodborne illness has emerged as an important public health threat worldwide due to its high morbidity rates and the emergence of antimicrobial resistance [6,7]. In this context, the European Food Safety Authority (EFSA) has estimated a disease burden and annual cost of 2.4 billion euros [8].

Campylobacteriosis is usually associated with a self-limiting illness characterized by diarrhea, nausea, abdominal cramps and bloody stools [9]. Only a minor percentage of cases are severe or fatal. Nevertheless, extraintestinal manifestations might cause long-term complications such as reactive arthritis, and Miller-Fisher or Guillain-Barré syndrome [10,11]. Despite the self-limiting course, certain populations such as children under 5 years of age, elderly patients, and immunocompromised patients might suffer severe infections and require antimicrobial treatment [9]. In this context, the World Health Organization (WHO) has included *Campylobacter* as a high priority pathogen [7] due to the worldwide emergence of strains with high level fluoroquinolone resistance [12,13].

In South America, there is limited data available on the prevalence of *C. jejuni* and *C. coli* in comparison to other enteric bacterial pathogens [3,14]. Furthermore, the incidence and number of cases reported varies substantially between countries, most probably due to differences in sensitivity of the diagnostic methods used in each surveillance program.

In Chile, the National Laboratory Surveillance Program (DTO. N° 7/2020) of the Public Health Institute of Chile (ISP) has reported a national incidence rate of campylobacteriosis

of 0.1 to 0.6/100.000 [15]. This is quite low compared to other high-income countries, where incidence rates are two orders of magnitude higher [3]. Nevertheless, recent reports suggest that the burden of campylobacteriosis in Chile has been greatly underestimated due to suboptimal diagnostic protocols in both public and private health centers [15]. A recent study showed a 10-fold increase of the detection rate of *Campylobacter* in human stool samples after changing from microscopic stool examination by Gram staining (the routine protocol in Chilean health centers) to culture-dependent techniques [16]. In another study from the same group, *Campylobacter* spp. emerged as the second most prevalent bacterial agents after diarrheagenic *Escherichia coli* when a multiplex PCR based assay (FilmArray® Gastrointestinal Panel) was used [17].

To date, only few reports have analyzed the genetic diversity and antimicrobial resistance profiles of clinical human isolates of *Campylobacter* in Chile [18–21]. Furthermore, these studies have either used a limited number of strains or lacked the resolution provided by sequence-based typing methods such as multilocus sequence typing (MLST). There is only one report that used whole-genome sequence data (wgMLST) to determine the genetic diversity of *C. jejuni* strains from the country, which was based on the first 3 genomes of Chilean *C. jejuni* strains. Therefore larger genomic epidemiology studies are needed [22].

We recently published the whole-genome sequences of 69 *C. jejuni* and 12 *C. coli* human clinical strains isolated from patients with enteric infection attending Clínica Alemana in Santiago, Chile, during a 2-year period (2017-2019) [1]. For 17 of the *C. jejuni* strains, we obtained complete genome assemblies [2]. This represents the largest collection of genomes from Chilean human clinical *Campylobacter* strains and includes the first *C. coli* genome in the country [1].

In the present study, we performed a genome-wide analysis of the genetic diversity, virulence potential, and antimicrobial resistance profiles of these 81 clinical strains. Importantly, we identified a high genetic diversity of both *C. jejuni* and *C. coli* strains, including 13 novel sequence types (STs) and alleles. Resistome analysis predicted a widespread resistance to fluoroquinolones, but a decreased resistance to tetracycline in comparison to the reported resistance from other regions of the world, in addition to the differential distribution of virulence factors, including plasmids and chromosomally encoded T6SS and T4SS.

METHODS

Genome data set, sequencing and reannotation

The genome sequence dataset analyzed in this study is listed in **Table S1** and was compiled from our recently published collection of human clinical strains of *C. jejuni* and *C. coli* from Central Chile [1,2] and available whole-genome sequences of *C. jejuni* and *C. coli* strains from public databases. We additionally included in this dataset the draft whole genome sequence of *C. jejuni* strain CFSAN096303, from the same collection of Clinica Alemana isolates which was sequenced for this study. The final genome composed of 2 contigs was obtained using a combination of long (nanopore) and short (Illumina, NextSeq) sequencing reads. Briefly, for the short reads the sequencing library was prepared using 1 ng DNA for the Nextera XT kit according to the manufacturer's instructions. The strain was sequenced using the NextSeq sequencer (Illumina). We used a NextSeq 500/550 high-output kit v2.5 (300 cycles) with 2 × 150-bp paired-end chemistry, according to the manufacturer's instructions, with >200X average coverage. The long reads were generated in a MinION sequencer (Nanopore Technologies, Oxford, UK). The sequencing library was prepared using the rapid barcoding sequencing kit (SQK-RBK004). The library for this strain was pooled with other 9 libraries and run in a FLO-MIN106 (R9.4.1) flow cell, according to the manufacturer's instructions, for 48 h. The run was base called live using default settings (Minknow v 19.06.8, Guppy v3.0.7). Default parameters were used for all software unless otherwise specified. The sequencing outputs for that run was 7.2 Gb (quality score, 11.46; N50, 6,000 bp; total reads, 569,349) for an estimated genome average coverage of 60 to 90 X. Reads less than 5,000 bp and quality scores of <7 were discarded for downstream analysis. To assemble the genome of *C. jejuni* strain CFSAN096303, the Illumina reads were managed with the CLC Genomics Workbench v9.5.2 (Qiagen), assessed for quality (Q, >30) with the quality control tool, and

trimmed (adapter trimming, quality trimming, and length trimming) with the trim sequences tool. The final complete genome sequence for this strain was obtained using a previously described pipeline [1], except that Flye was used instead of CANU for long read de novo assembling. The genomes were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP, http://www.ncbi.nlm.nih.gov/genome/annotation_prok). Default parameters were used for all software unless otherwise specified. The NextSeq data was deposited in NCBI under accession number SRR10859573. The nanopore raw data was deposited in NCBI under accession number SRR11786978. The final draft genome was deposited under accession number WXZQ01000000. Fasta files of all draft genomes from the collection of strains from Chile were reordered to the reference genomes of the *C. coli* aerotolerant strain OR12 (accID GCF_002024185.1) and *C. jejuni* strain NCTC 11168 (accID GCF_000009085.1) using the Mauve Contig Mover (MCM) from the Mauve package [23]. The ordered genomes were reannotated using Prokka [24] with the same reference files mentioned above and by forcing GenBank compliance.

MLST and cgMLST analysis:

For MLST and cgMLST, genome assemblies ere mapped against an MLST scheme based on seven housekeeping genes [25] and the 1,343-locus cgMLST scheme [26], using Ridom SeqSphere software (Münster, Germany) [27]. *C. hepaticus HV10* (accID GCF-001687475.2)*, C. upsaliensis DSM 5365* (accID GCF-000620965.1), *C. lari RM2100* (accID GCF-000019205.1), *C. jejuni NCTC 11168* (accID GCF-000009085.1*), C. jejuni doylei 269.97 (*accID GCA-000017485.1*),* aerotolerant *C. coli OR12 (*accID *GCF-002024185.1), C. coli BIGS0008 (*accID *GCA-000314165.1), C. coli 76339 (*accID GCA-000470055.1*),* and *C. coli H055260513* (accID GCA-001491555.1) were used as reference genomes. Phylogenetic comparison of the 81 *Campylobacter* genome

sequences was performed using a neighbor-joining (NJ) tree based on a distance matrix of the core genomes of all the strains. STs and clonal complexes (CCs) were determined after automated allele submission to the PubMLST server for *Campylobacter* MLST (https://pubmlst.org/campylobacter/). Additionally, to compare the isolates with previously described *C. jejuni* and *C. coli* isolates worldwide, we performed a similar phylogenetic analysis using the NCBI database including the isolates reported for the whole Latin American region. Further annotations and visualizations were performed using iTOL v.4 [28].

Virulome, resistome and comparative genomic analysis

A local BLAST database was constructed using the fasta files of all sequenced genomes and the makeblastdb program from BLAST (NCBI) [29]. Subsequently, the database was screened for the nucleotide sequence of known pathogenicity genes using BLASTn (version 2.8.1) [29]. A threshold of 90% of the original sequence length and 90% identity was used to select positive matches. A fasta file containing the nucleotide sequence of *Campylobacter* pathogenicity genes screened in this work can be found at https://doi.org/10.5281/zenodo.3925206. Mutations in *gyrA* and 23S rRNA were searched as indicative of resistance to fluoroquinolones and macrolides, respectively. The sequences of both genes were screened by BLAST in the local BLAST database and sequences were aligned to the reference gene from strain Campylobacter jejuni ATCC 33560 using ClustalX [30]. We then manually searched for mutation C257T in *gyrA* and mutations A2058C and A2059G in the genes for 23S RNA. Antimicrobial resistance genes were additionally screened in each *Campylobacter* genome by means of the ABRicate pipeline [31], using the included Resfinder [32], CARD [33], ARG-ANNOT [34] and NCBI ARRGD [35] databases. BLAST-based genome comparison analysis and visualization of genetic clusters were performed using EasyFig [36].

Pangenome and plasmid screening analysis

Annotated genome sequences from all sequenced genomes were compared using Roary [37], setting a minimum percentage identity of 90% for BLASTp. Visualization of genes shared between genomes was performed using roary_plots.py. Additionally, the file gene_presence_absence.csv [https://github.com/sanger-pathogens/Roary/tree/master/contrib/roary_plots] produced by Roary was further analyzed using a spreadsheet program to find genes that are shared by subgroups of isolates. Using this information Venn diagrams were constructed using the tool available at http://bioinformatics.psb.ugent.be/webtools/Venn/. The screen for plasmids was performed on the FASTQ files of each sequencing reaction using PlasmidSeeker [38] based on the default bacterial plasmid database. Additional searches were performed using an inhouse database containing known plasmids from *C. coli* or *C. jejuni*. The fasta file for in-house databases can be found in https://doi.org/10.5281/zenodo.3925206. Pangenome BLAST analysis was performed using the GView server (https://server.gview.ca/) [39].

Results and Discussion

Phylogenetic analysis of clinical Campylobacter strains

cgMLST analysis showed high genetic diversity among the 69 *C. jejuni* strains (**Figure 1A**), as shown by the 14 CCs and 31 distinct STs identified. Almost 93% (n=64) of the *C. jejuni* strains belonged to one of the CCs previously described for this species. Among our strains, CC-21 was the most common (37.6%) and diverse (5 distinct STs) (**Figure 1A, Table S1**). This is consistent with other reports, which describe CC-21 as the largest and most widely distributed clonal complex representing 18.9% of all *C. jejuni* isolates submitted to the PubMLST database. ST-1359 was the most prevalent ST of CC-21 (48%) of all strains in this study (17.4%) (**Figure 1A and Table S1**). The high prevalence of ST-1359 was unexpected, since it is infrequent within the PubMLST database (0.06% by February 2020). However, ST-1359 has been recognized as a prominent ST in Israel [40]. Other STs within CC-21 included ST-50 (8.7%) and ST-21 (1.45%), which were predominant in reports from Israel, Belgium, and the United Kingdom [40–42] as well as worldwide in PubMLST (4.1% and 4.3% respectively). ST-45, the second most prevalent sequence type worldwide worldwide (4.2%), was not identified among our strains. In addition to CC-21, we identified high prevalence rates of CC-48 (13%) and CC-52 (7.3%). ST-475 from CC-48 was the second most prevalent ST (11.6%) identified (**Figure 1A**).

There are only two previous reports of MLST data of clinical *C. jejuni* strains from Chile [18,22]. Both reports identified four major CCs and STs, including CC-21 (ST-50), CC-48 (ST-475), CC-257 (ST-257), and CC-353 (ST-353). Our study is consistent with these reports, except for the high prevalence of ST-1359 (CC-21), which has not been previously reported in Chile. Further epidemiological information is needed to determine if the high

prevalence of these four CCs is specific to Chile and to determine if the high prevalence of ST-1359 strains in our report is an isolated finding or if it represents an emergent ST in Chile.

C. coli is divided into three genetic clades [43,44]. Clade 1 is subdivided into two major clonal complexes, CC-828 and CC-1150, and often isolated from farm animals and human cases of gastroenteritis [44]. Clades 2 and 3 lack clonal complex substructures and are mainly isolated from environmental sources [43,44]. cgMLST analysis of the 12 *C. coli* strains from our collection revealed that 83% (10/12) of the strains belonged to clade 1, while 17% (2/12) belonged to clade 3 and none to clade 2. Most clade 1 strains (8/10) belonged to CC-828 and included four previously described STs (828, 829, 1173, and 1556) and one novel ST (10201). Only one strain from clade 1 belonged to the usually common CC-1150 (strain CFSAN093253 from ST-10203), and another strain could not be assigned to any clonal complex (strain CFSAN096322 from ST-10202). Both clade 3 strains belonged to ST-10204. Overall, clade 1 strains from CC-828 were most prevalent, which is consistent with the worldwide distribution of this species. Interestingly, despite the low incidence of cases of gastroenteritis associated with *C. coli*, in the 2-year period of our study (only 12 strains in total), we were able to identify strains from the uncommon clade 3. Considering that these are the first strains of *C. coli* described from Chile and considering the size of our study, further genomic epidemiological studies are needed to fully grasp the diversity of the strains from this country.

A global cgMLST analysis comparing our 81 *Campylobacter* genomes with other *C. jejuni* and *C. coli* genomes available from the NCBI database was performed. The strains of our collections from Central Chile showed great diversty and did not group with any existing clusters (**Figure 1B)** (the interactive tree and metadata are available in Microreact, see

data summary).

Pangenome analysis of Campylobacter strains

To identify the genetic elements that are shared and distinct among the Chilean *C. jejuni* and *C. coli* strains, we performed pangenome analysis of the 81 strains from both species (**Figure 2**). The analysis identified a pangenome of 6609 genes for both species and identified the core genes shared between *C. jejuni* and *C. coli* (532 genes) and the core genome of each species (1300 genes for *C. jejuni* and 1207 genes for *C. coli*). In addition, the analysis was able to identify the differences among the core and accessory genomes of *C. coli* strains from clade 1 and clade 3. *C. coli* strains from clade 1 harbored 340 core genes absent from clade 3 core genes, which in turn, harbored 159 core genes not present in clade 1 core genes (**Figure 2**). The estimated core genome for *C. jejuni* in our study (1300 genes) was close to the 1343 core genes identified in the development of the cgMLST scheme of *C. jejuni* [25] and in agreement with recent reports [45–47] (**Table S2**). In addition, a significant variation in the accessory genome between *C. jejuni* and *C. coli* strains was identified, confirming the high genetic variability of *Campylobacter*. Accessory genes that are common to a particular cluster seem to contribute to modifications of carbohydrates and flagella as well as antibiotic resistance. There is also a proportion of genomes harboring phage- and type IV and VI secretion system-related genes (see below).

Genomic analysis of the resistome of Chilean clinical Campylobacter strains.

Although most *Campylobacter* infections are self-limiting and do not require antibiotic treatment, the use of antibiotics is indicated for patients with persistent and severe

gastroenteritis, patients with extraintestinal infections, and patients who are immunocompromised [3].

To determine the resistome of the *C. jejuni* and *C. coli* strains, we performed an *in silico* analysis to identify genes associated with antimicrobial resistance (**Figure 3**). We screened each *Campylobacter* genome by means of the ABRicate pipeline [31], using the Resfinder, CARD, ARG-ANNOT and NCBI ARRGD databases, to identify antimicrobial resistance genes. We additionally performed sequence alignments to identify point mutations in *gyrA* and 16S rRNA genes that have been previously described to confer antimicrobial resistance.

The *cmeABC* operon was present in all *C. jejuni* and *C. coli* strains analyzed. This operon encodes a common multidrug efflux pump characterized in different *Campylobacter* species, which mainly confers resistance to fluoroquinolones and macrolides [48,49]. Additionally, the point mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, leading to the T86I substitution in GyrA, was identified in 54.3% of the strains. This mutation is the most frequently reported mutation worldwide and confers high levels of resistance to fluoroquinolones [48,49]. The high percentage of *C. jejuni* strains with this mutation is consistent with recent studies from Chile, reporting ciprofloxacin resistance in 30-60% of *C. jejuni* strains [18,20–22]. While Porte *et al.* reported that most resistant strains were associated with ST-48 and ST-353, in our study, there was no particular association of this mutation specific STs. However, it is important to note that 100% of strains belonging to the most frequent ST described in this work (ST-1359) harbored the mutation in the QRDR region. , while most ST-475 isolates (the second most prevalent ST) not have this mutation. Although fluoroquinolones are not first choice antimicrobial for human campylobacteriosis, these high levels of resistance might be a

consequence of their broad use in animal husbandry. Fernandez and Perez-Perez concluded that the primary source of transmission of resistant *Campylobacter* strains to humans in Latin America is related to the consumption of animal-origin food and interactions with environmental animal sources [50]. However, further studies are needed to obtain a better understanding of the relevance of animal production practices in the dissemination of antibiotic resistance genes among Chilean *Campylobacter* strains.

Regarding macrolide resistance, neither mutation of the 23S rRNA gene nor presence of the *ermB* gene was detected, which is consistent with previous reports on Chilean *C. jejuni* strains [18,22]. The low incidence of resistance against this group of antibiotics (especially azithromycin and erythromycin) makes them the first choice for treatment of *Campylobacter* infections [51]. However, further studies are necessary to analyze a higher number of *C. coli* strains. This is particularly important given that according to EFSA, macrolide resistance has been mostly detected in this species [52].

In terms of tetracycline resistance, 22.2% of the strains harbored the *tetO* gene, which is the main genetic factor mediating tetracycline resistance in *Campylobacter* spp [53]. This gene can be harbored on a plasmid or chromosomally encoded and is widespread among *Campylobacter* strains worldwide [53]. Recently, the European Union (EU) reported very high levels of tetracycline resistance in *C. jejuni* strains isolated from humans (45.4%) and even higher levels of resistance in *C. coli* (68.3%). This antibiotic is widely used, especially in the poultry industry worldwide [54], and might serve as an important reservoir for resistant strains [55]. In fact, Lapierre *et al*. reported that 32.3% of *C. jejuni* isolates from poultry in Chile were resistant to tetracycline [21]. Our data are consistent with a recent report from Chile [21] that found that 22% of *C. jejuni* strains of clinical origin were tetracycline resistant, which suggests that Chilean strains of *Campylobacter* have lower

levels of tetracycline resistance than strains from other countries. This highlights the need for a permanent surveillance program to implement control measures for tetracycline usage in animal production to avoid an increase in tetracycline resistance levels in the human population. Finally, we also found a high proportion of strains (79%) harboring the blaOXA-61 gene among *C. jejuni* and *C. coli* strains. This genetic marker is associated with β-lactam resistance and has high prevalence in *Campylobacter* isolates worldwide [56,57].

Presence and diversity of virulence gene content among Chilean Campylobacter strains

The ability of *Campylobacter* to cause disease in humans is thought to be multifactorial, as it lacks several of the key virulence factors used by other enteric bacterial pathogens to cause disease (e.g., type III secretion systems). Nevertheless, there are several genes and loci that are associated with the virulence and pathogenic potential of *Campylobacter*, even though their role in the development of campylobacteriosis is not fully understood [58]. Furthermore, most of our knowledge regarding the virulence potential of *Campylobacter* strains comes from studies performed in *C. jejuni*; therefore, information regarding the genetic determinants that contribute to the virulence of *C. coli* strains is still lacking.

To determine the virulence gene content of the *C. jejuni* and *C. coli* strains, we first assembled a list of 220 potential virulence genes, including genes described in the Virulence Factor Database [59] and genes reported to contribute to the virulence of *Campylobacter* in the literature [38,60–63]. These genes were grouped into five distinct categories (adhesion and colonization, invasion, motility, secretion systems and toxins)

and were used to screen each of the 81 genomes using BLASTn**.** The distribution of the major virulence factors described for *C. jejuni* and *C. coli* is shown in **Figure 4** (see **Figure S1** and **Table S3** for the full list).

Most of the described factors involved in adhesion and colonization (*cadF*, *racR*, *jlpA, pdlA, dnaJ, aphC*) were prevalent among the *C. jejuni* strains and were not associated with any particular STs (**Figure 4**, **Table S3**). This finding is consistent with other reports indicating that the prevalence of these factors in clinical strains is very high [63] and shows the relevance of adherence in the pathogenicity of *C. jejuni*. The genes responsible for the production of the CDT toxin (*cdtA*, *cdtB* and *cdtC*) were also found in most strains of *C. jejuni* and in every *C. coli* strain tested. The *cdtA* and *cdtB* genes were not detected in the *C. jejuni* strain CFSAN093229. In contrast, the *ciaB* gene was identified only in *C. jejuni* (in 98.7% of the strains) (**Figure 4**). The product of this gene is involved in cecal colonization in chickens and in translocation into host cells in *C. jejuni* [64,65]. The absence of this gene in *C. coli* might suggest that it does not play a crucial role in the pathogenesis of this *Campylobacter* species. The major differences among *C. jejuni* strains were observed for genes located at the *cps* locus (Cj1413c -Cj1442) involved in capsule polysaccharide synthesis, the LOS locus (C8J1074-C8J1095) involved in lipooligosaccharide synthesis, a small set of genes in the O-linked flagellin glycosylation island (Cj1321-Cj1326) and the T6SS and T4SS gene clusters (**Figure 4**, **Figure S1 and Table S3**).

Most virulence genes described for *C. jejuni* were absent in *C. coli* clades 1 and 3 strains, highlighting the need for a better understanding of how *C. coli* strains cause the disease. However, *C. coli* strains carried most of the genes known to be associated with invasiveness in *C. jejuni*, including the *iamb, flaC* and *ciaB* genes. Both *C. coli* clades also

harbored some genes associated with other steps of infection such as the *cdt* toxin gene and most of the flagellar biosynthesis and chemiotaxis-related genes described for *C. jejuni* (**Figure S1**). The high prevalence of the *iamb* gene in *C. coli* has also been observed in other studies, but its relevance in pathogenicity remains unclear [66].

Distribution of the T4SS and T6SS gene clusters.

To date, there is only one T6SS cluster described in *Campylobacter* [60], and it has been linked to a wide variety of phenotypes, including host cell adherence, cellular invasion and resistance to bile salt, and oxidative stress [67–69]. In addition, *Campylobacter* T6SS mutant strains exhibit colonization defects in both mouse [67] and chicken [69] *in vivo* infection models. The *Campylobacter* T6SS gene cluster is often encoded within the CjIE3 conjugative element in the chromosome of *C. jejuni* [61], but it can also be found in plasmids [70,71]. Our analysis identified the T6SS gene cluster in 9 *C. jejuni* strains and in 1 *C. coli* strain from clade 1 (**Figure 4**). As shown in **Figure 5A**, whenever present the genetic structure and the sequence identity of this cluster was highly conserved among *C. jejuni* and *C. coli* strains. For strains with genomes that were not closed, we were not able to confirm whether the *tssI* gene is located within the genetic context of the of the T6SS cluster, since it was not assembled in the same contig as the rest of the cluster [70].

Since most of our sequenced strains correspond to draft genome assemblies, we could not determine whether the T6SSs were plasmid or chromosomally-encoded in most of them. However, in two T6SS-positive strains with closed genomes (CFSAN093238 and CFSAN093227), T6SS was inserted in the chromosomal CjIE3 element, while in strain CFSAN093246, it was plasmid-encoded. Sequence-based analysis of this plasmid showed a high degree of identity with plasmid pMTVDDCj13-2 (**Figure 6A**), which

encodes a complete T6SS gene cluster and was recently sequenced from the MTVDSCj13 strain of *C. jejuni* [71].

No correlation was found between the T6SS gene cluster and a particular ST or clonal complex. This is different from what has been described in other countries, such as Israel, where most T6SS-positive strains belong to ST-1359 [40]. Although ST-1359 was the most common ST described in this study, none of the strains carried genes encoding T6SS.

To date, two distinct T4SSs have been described in *Campylobacter*. One of them is encoded in the pVir plasmid of *C. jejuni* and is homologous to the T4SSs from *Helicobacter pylori* [72]. The pVir T4SS has been linked to the virulence of *C. jejuni*. T4SS mutants show reduced invasion capacity of INT407 cells and decreased diarrhea-inducing ability in a ferret model [62,73]. The second T4SS is encoded in the pTet and pCC31 family of plasmids of *C. jejuni* and *C. coli*. This distinct T4SS is involved in bacterial conjugation and is not directly linked to the virulence of *C. jejuni* and *C. coli* strains [74].

We identified a T4SS gene cluster homologous to this second T4SS in 12 strains of *C. jejuni* and 1 strain of *C. coli* clade 1 (**Figure 4**). It harbored the 16 core genes and was correctly assembled in individual contigs in 11 strains, which allowed the analysis of the genetic context of the cluster. As shown in **Figure 5B**, in each strain, the T4SS cluster had a conserved genetic structure. One of the main differences between the T4SS gene clusters encoded in plasmids pTet and pCC31 is the sequence divergence of the *cpp33* gene [74]. Only the *cp33* present in strain CFSAN096321 showed high identity with the gene harbored in the pTet plasmid. T4SS gene clusters from every other *C. jejuni* and *C. coli* strain possessed high identity to the *cp33* encoded in pCC31 plasmid.

As shown in **Figure 6B**, plasmids pCFSAN093226 and pCFSAN096297 share significant similarity with both the pCC31 and pTET plasmids beyond the T4SS gene cluster. For all other strains, BLASTn analysis of each of our draft genome assemblies identified most of the plasmid genes found in pCFSAN093226, pCFSAN096297 and pTet-like plasmids, suggesting that all the T4SS gene clusters identified in these strains are encoded in plasmids as well.

Conclusions:

The genomes reported in the current study represent 96.4% of the sequenced Chilean *Campylobacter* strains and 36.8% of the strains from the South American region. Taken together, the results of this study provide valuable epidemiological data and relevant information about the population structure, virulence-associated genes and AMR profiles of an important number of clinical *Campylobacter* isolates. Further studies are needed to determine the dynamics of transmission of pathogenic *Campylobacter* from animals to humans and to monitor the emergence of new virulence and antimicrobial resistance markers that might represent a threat to the human population. Finally, we expect that these actionable public health data will support the design of strengthened surveillance programs, which will be essential for the development of an effective integrated health system in Chile.

AUTHOR CONTRIBUTIONS

Veronica Bravo: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Data curation, Writing-Original Draft and review and editing.

Assaf Katz: Methodology, Formal analysis, Investigation, Visualization, Data Curation, Resources, Writing-Original Draft and review and editing.

Carmen Varela: Resources, Writing - Review and editing

Lorena Porte: Resources, Writing - Review and editing.

Thomas Weitzel: Resources, Writing - Review and editing.

Narjol Gonzalez-Escanola: Conceptualization, Methodology, Formal analysis, Resources, review and editing final manuscript

Carlos J Blondel: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Resources, Data curation, Writing - Review and editing, Funding acquisition, Supervision and Project administration.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Figure 1. A) Phylogenetic analysis of *C. jejuni* and *C. coli* strains from Central Chile. The phylogenetic tree is based on cgMLST analyses performed with RIDOM SeqSphere^{+} and visualized by iTOL. Sequence types (STs) are shown in colored boxes for each of the *Campylobacter* strains, and the tree branches have been color coded to highlight the *C. jejuni* and *C. coli* strains from clades I, II and III. **B)** Global cgMLST analysis and distribution of clonal complexes of clinical *C. jejuni* and *C. coli* strains. Strains from this study are highlighted in red. cgMLST analysis performed by SeqSphere+ and visualized by iTOL.

Figure 2. Visualization of the pangenome of the 69 *C. jejuni* and 12 *C. coli* strains from Chile. Pangenome analysis was performed using Roary. The core and accessory genomes are highlighted with red and black brackets, respectively. The cgMLST phylogenetic tree of Figure 1A is shown on the left. A Venn diagram highlighting the number of core genes present in *C. jejuni* and *C. coli* strains from clades I and III is shown on the right.

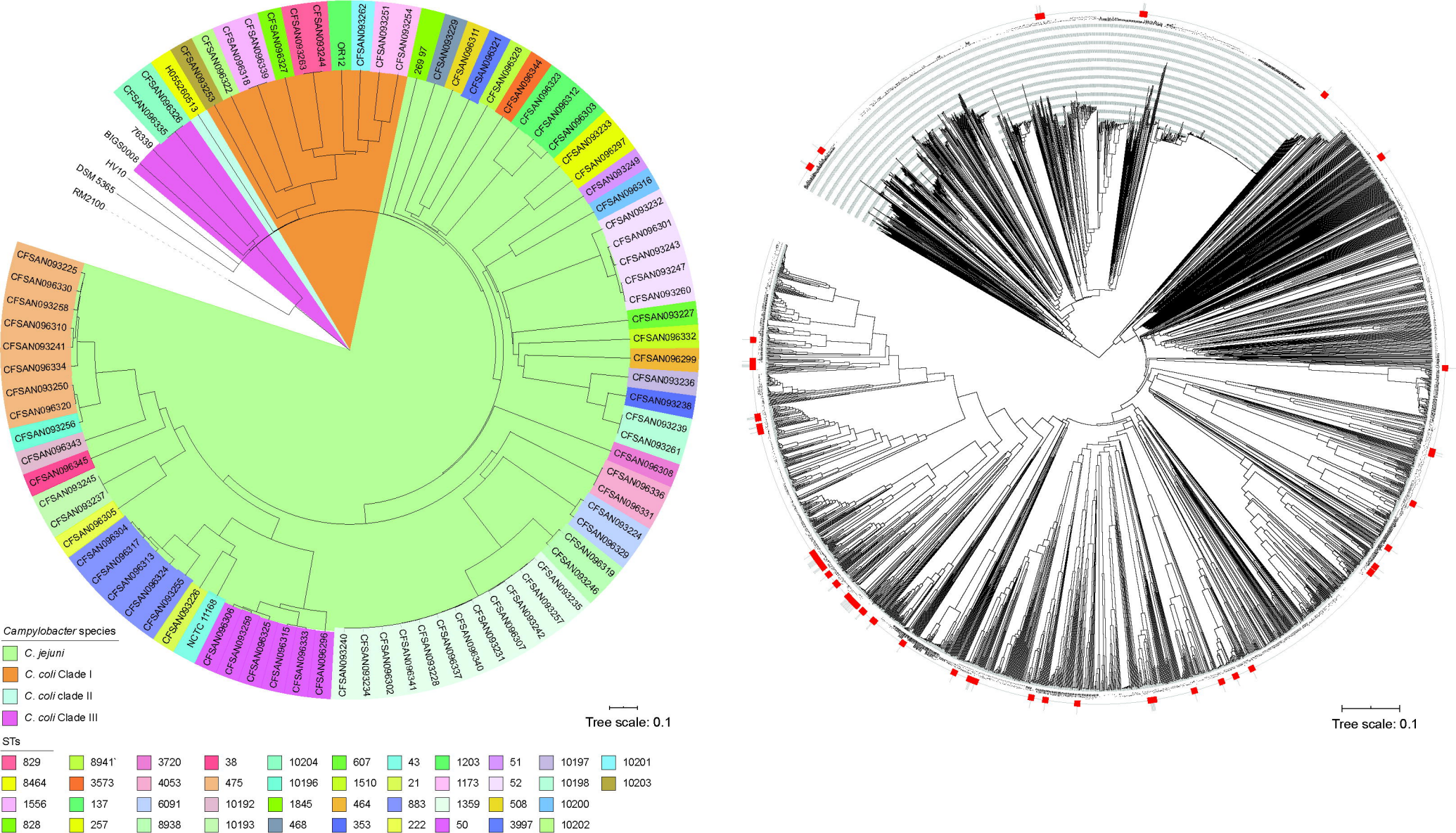
Figure 3. Distribution of antimicrobial resistance genes in *C. jejuni* and *C. coli* strains from Chile. Binary heatmaps show the presence and absence of antimicrobial resistance genes. Colored cells represent the presence of genes, and blank cells represent the absence of genes. The phylogeny of *C. jejuni* and *C. coli* strains is the same as that in Figure 1. Strain names are color coded to highlight phylogenetic groups: *C. jejuni* strains (light green), *C. coli* clade I strains (orange) and *C. coli* clade III strains (magenta).

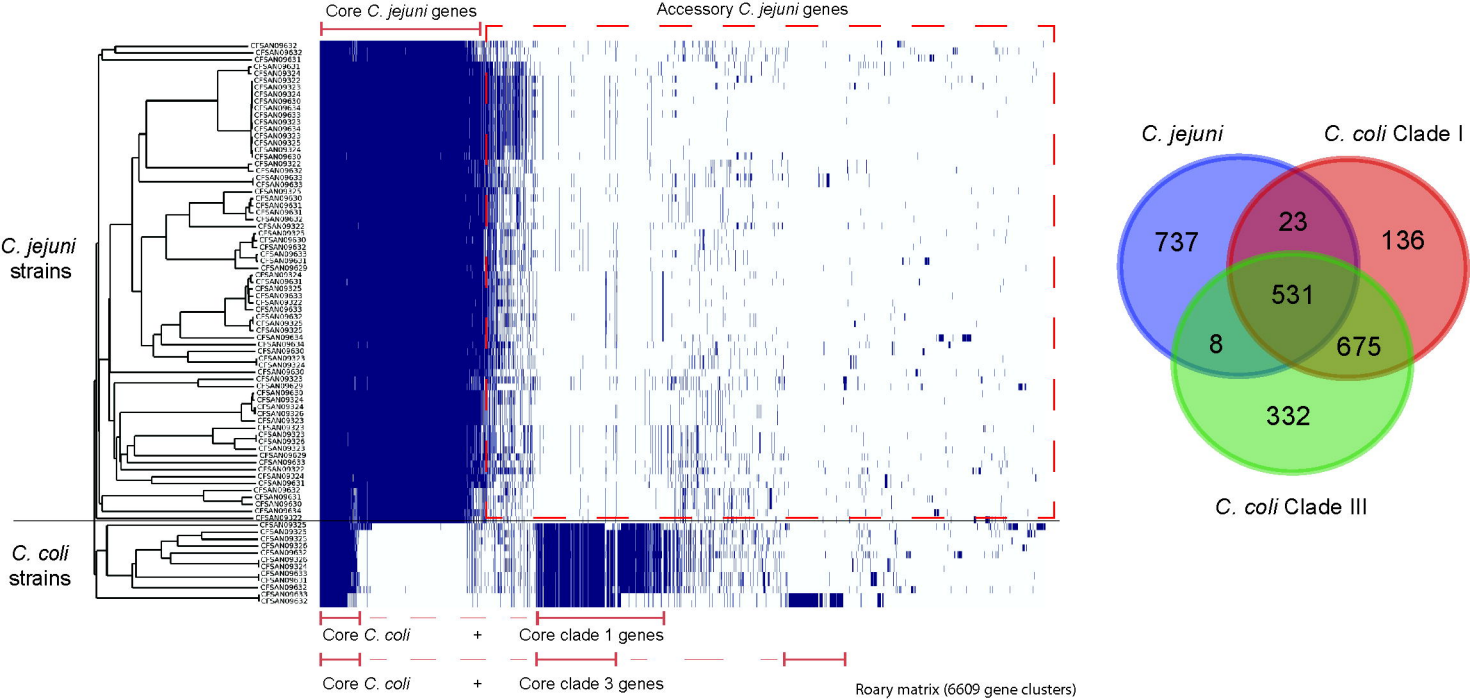
Figure 4. Distribution of virulence-related genes in *C. jejuni and C. coli* strains. Binary heatmaps show the presence and absence of virulence genes linked to bacterial adhesion (red), invasion (blue), toxins (purple) and specialized protein secretion systems (green). Colored cells represent the presence of genes, and blank cells represent the absence of genes. The phylogeny of *C. jejuni* and *C. coli* strains is the same as that in Figure 1. Strain names are color coded to highlight *C. jejuni* strains (green), *C. coli* clade I strains (orange) and *C. coli* clade III strains (purple).

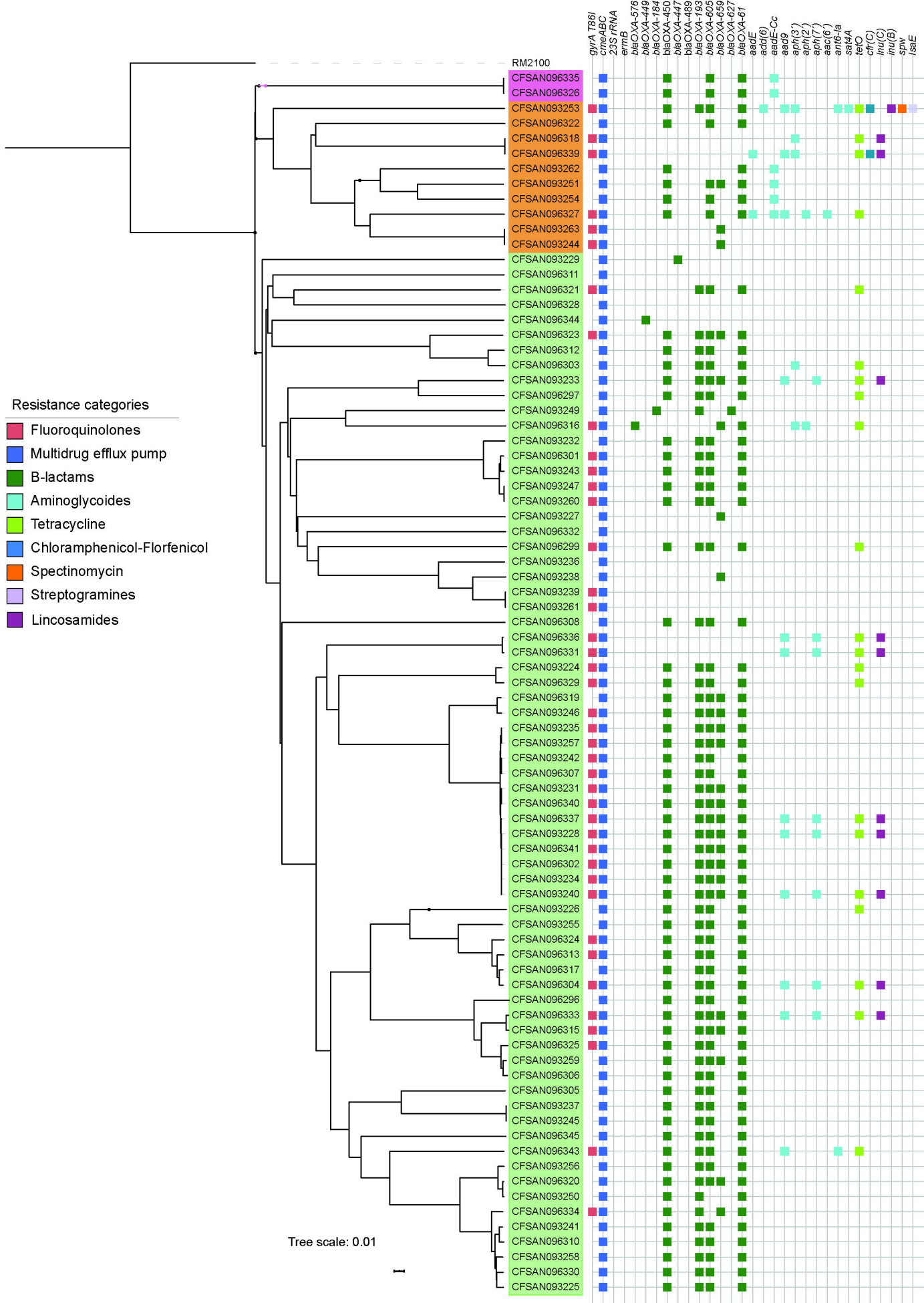
Figure 5. Comparative genomic analysis of the T6SS and T4SS gene clusters identified in clinical *C. jejuni* and *C. coli* strains. **A)** Genetic organization of the T6SS gene clusters of *C. jejuni* and *C. coli* strains (bold) compared to the cluster of *C. jejuni* strain 108. The genes *tagH*, *tssD*, *tssB* and *tssC* are shown in color. **B)** Genetic organization of the T4SS gene clusters identified in clinical *C. jejuni* strains in comparison to the T4SS gene clusters of the plasmids pTet and pCC31. The *cp33* gene is highlighted in orange. BLASTn alignments were performed and visualized using EasyFig.

Figure 6. Comparative genomic analysis of plasmids encoding T6SS **(A)** and T4SS **(B**) gene clusters in clinical *C. jejuni* strains. Pangenome BLAST analysis of plasmids was performed using the Gview server. T6SS and T4SS gene clusters are highlighted in red brackets.

Figure S1. Distribution of the presence (red) and absence (blue) of virulence-related genes in *C. jejuni* and *C. coli* strains from the study.


Campylobacter species
C. jejuni
C. coli Clade I
C. coli clade II
C. coli Clade III
Tree scale: 0.1
STs
829
8941`
3720
38
10204
607
43
1203
51
10197
10201
8464
3573
4053
475
10196
1510
21
1173
52
10198
10203
1556
137
6091
10192
1845
464
883
1359
508
10200
828
257
8938
10193
468
353
222
50
3997
10202
Tree scale: 0.1


Core C. jejuni genes
Accessory C. jejuni genes
C. jejuni strains
C. coli strains
Core C. coli + Core clade 1 genes
Core C. coli + Core clade 3 genes
Roary matrix (6609 gene clusters)
C. jejuni
C. coli Clade I
C. coli Clade III
737
23
136
531
8
675
332


gyrA T86I
cmeABC
23S rRNA
ermB
blaOXA-576
blaOXA-449
blaOXA-184
blaOXA-450
blaOXA-447
blaOXA-489
blaOXA-193
blaOXA-605
blaOXA-659
blaOXA-627
blaOXA-61
aadE
add(6)
aadE-Cc
aad9
aph(3')
aph(2')
aph(7')
aac(6')
ant6-Ia
sat4A
tetO
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spw
lsaE
RM2100
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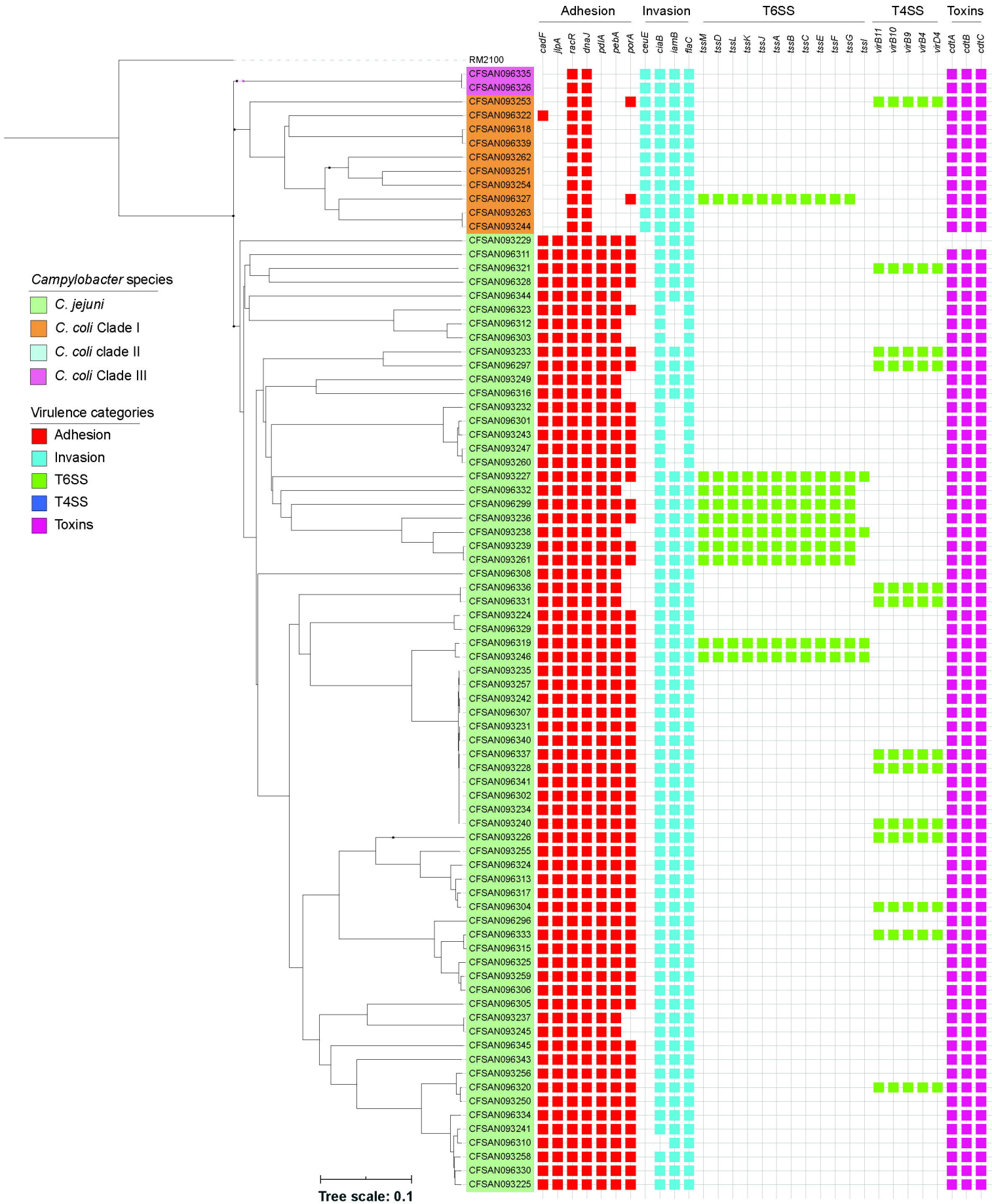
Resistance categories
Fluoroquinolones
Multidrug efflux pump
B-lactams
Aminoglycoides
Tetracycline
Chloramphenicol-Florfenicol
Spectinomycin
Streptogramines
Lincosamides
Tree scale: 0.01


Adhesion
Invasion
T6SS
T4SS
Toxins
cadF
jlpA
racR
dnaJ
pdlA
pebA
porA
ceuE
claB
iamB
flaC
tssM
tssD
tssL
tssK
tssJ
tssA
tssB
tssC
tssE
tssF
tssG
tssI
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virB10
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virD4
cdtA
cdtB
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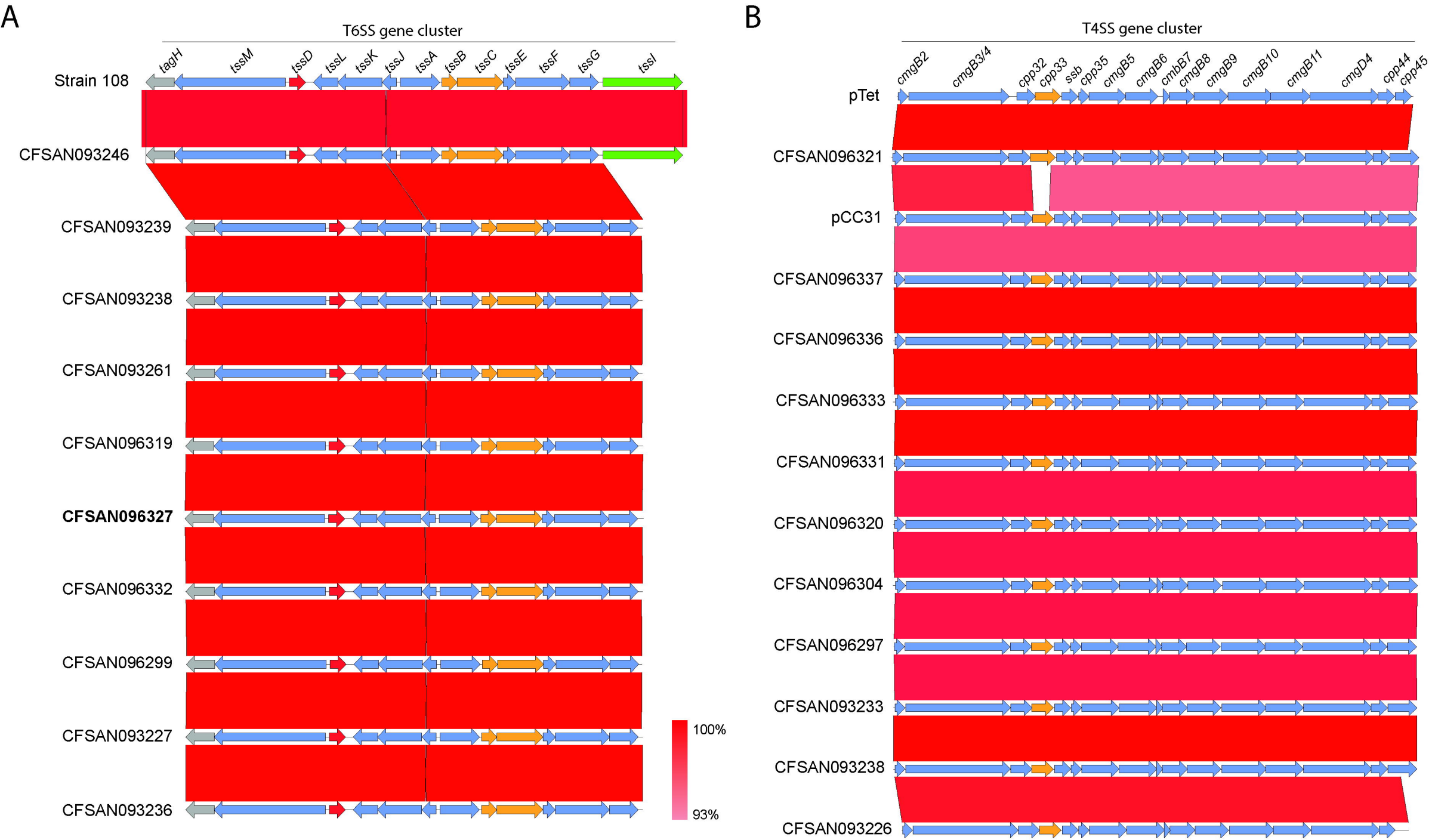
Campylobacter species
C. jejuni
C. coli Clade I
C. coli clade II
C. coli Clade III
Virulence categories
Adhesion
Invasion
T6SS
T4SS
Toxins
Tree scale: 0.1


A
T6SS gene cluster
tagH
tssM
tssD
tssL
tssK
tssJ
tssA
tssB
tssC
tssE
tssF
tssG
tssI
Strain 108
CFSAN093246
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CFSAN093238
CFSAN093261
CFSAN096319
CFSAN096327
CFSAN096332
CFSAN096299
CFSAN093227
CFSAN093236
100%
93%
B
T4SS gene cluster
cmgB2
cmgB3/4
cpp32
cpp33
ssb
cpp35
cmgB5
cmgB6
cmbB7
cmgB8
cmgB9
cmgB10
cmgB11
cmgD4
cpp44
cpp45
pTet
CFSAN096321
pCC31
CFSAN096337
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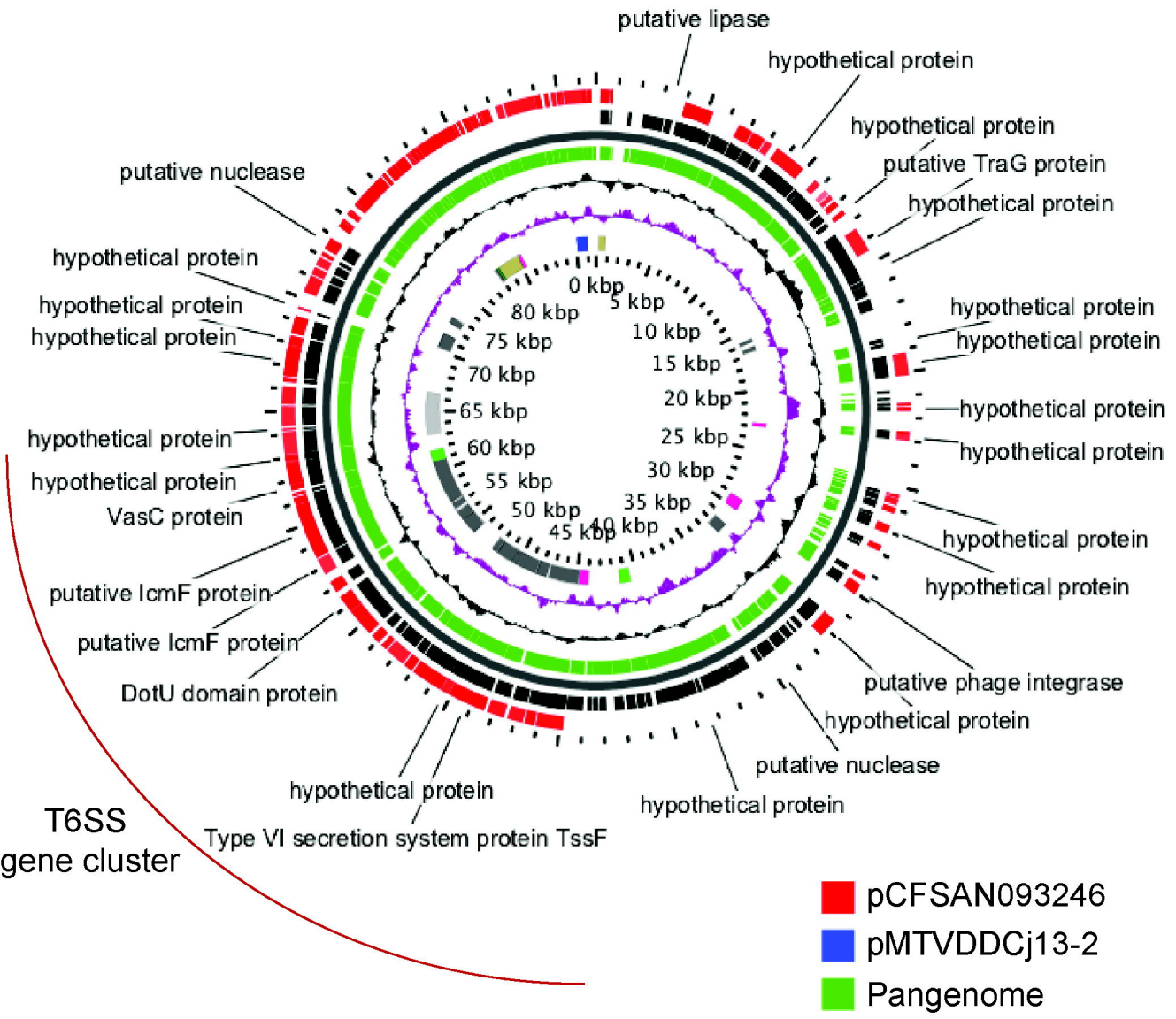
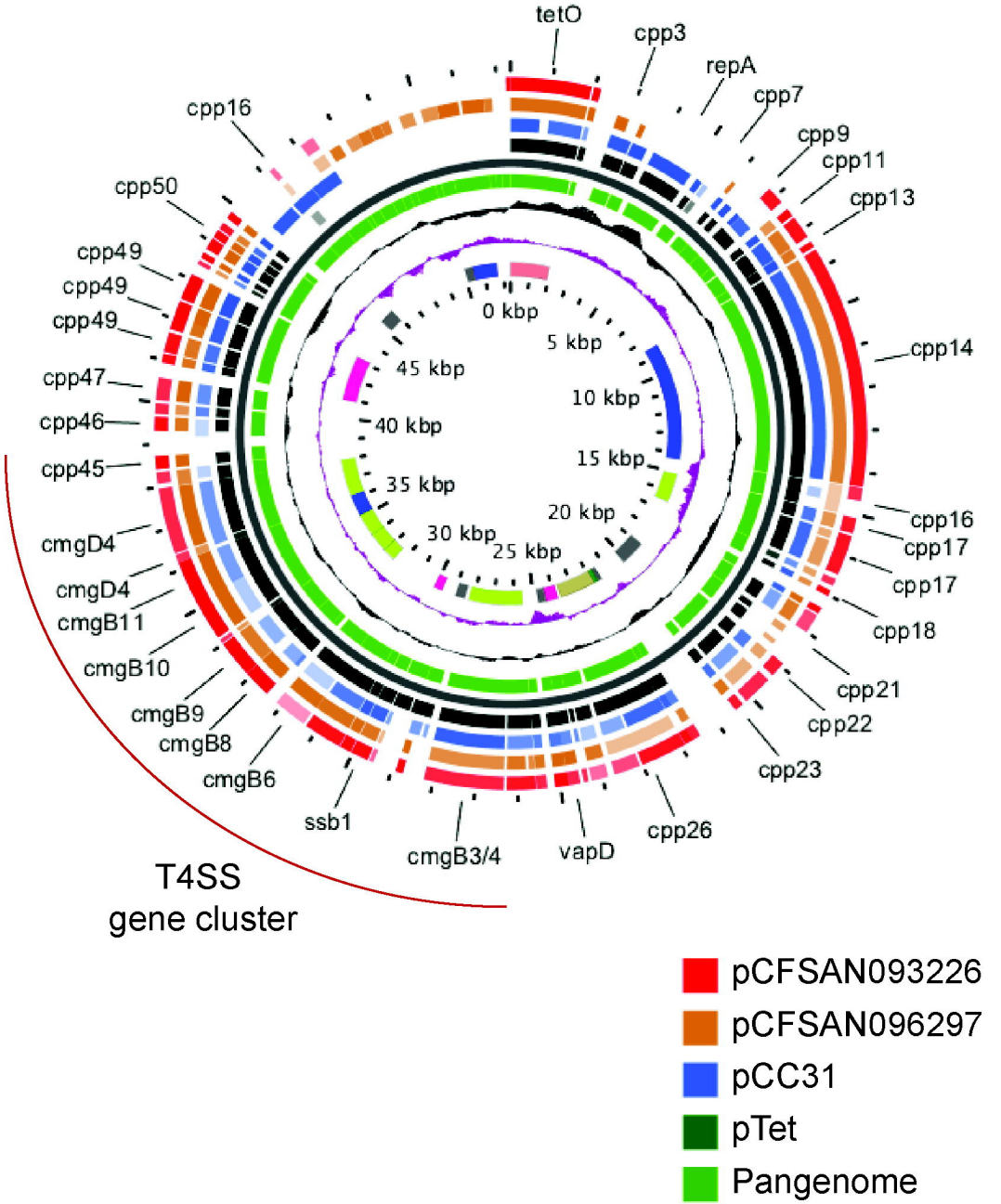

putative lipase
hypothetical protein
hypothetical protein
putative TraG protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
putative phage integrase
hypothetical protein
putative nuclease
hypothetical protein
hypothetical protein
Type VI secretion system protein TssF
DotU domain protein
putative IcmF protein
putative IcmF protein
VasC protein
hypothetical protein
hypothetical protein
hypothetical protein
hypothetical protein
putative nuclease
0 kbp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
50 kbp
55 kbp
60 kbp
65 kbp
70 kbp
75 kbp
80 kbp
T6SS
gene cluster
pCFSAN093246
pMTVDDCj13-2
Pangenome


tetO
cpp3
repA
cpp7
cpp9
cpp11
cpp13
cpp14
cpp16
cpp17
cpp17
cpp18
cpp21
cpp22
cpp23
cpp26
vapD
cmgB3/4
ssb1
cmgB6
cmgB8
cmgB9
cmgB10
cmgB11
cmgD4
cmgD4
cpp45
cpp46
cpp47
cpp49
cpp49
cpp49
cpp50
cpp16
0 kbp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
T4SS
gene cluster
pCFSAN093226
pCFSAN096297
pCC31
pTet
Pangenome