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Whole-Genome Sequence Analysis of Multidrug-Resistant *Campylobacter* Isolates: a Focus on Aminoglycoside Resistance Determinants

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ABSTRACT A whole-genome sequencing (WGS) approach was conducted in order to identify the molecular determinants associated with antimicrobial resistance in 12 multidrug-resistant *Campylobacter jejuni* and *Campylobacter coli* isolates, with a focus on aminoglycoside resistance determinants. Two variants of a new aminoglycoside phosphotransferase gene [*aph*(2″)-*Ii*$_1$ and *aph*(2″)-*Ii*$_2$] putatively associated with gentamicin resistance were found. In addition, the following new genes were identified for the first time in *Campylobacter*: a lincosamide nucleotidyltransferase gene [*lnu*(G)], likely associated with lincomycin resistance, and two resistance enzyme genes (*spw* and *apmA*) similar to those found in *Staphylococcus aureus*, which may confer spectinomycin and gentamicin resistance, respectively. A C1192T mutation of the 16S rRNA gene that may be involved in spectinomycin resistance was also found in a *C. coli* isolate. Genes identified in the present study were located either on the bacterial chromosome or on plasmids that could be transferred naturally. Their role in aminoglycoside resistance remains to be supported by genetic studies. Regarding the other antimicrobial agents studied, i.e., ampicillin, ciprofloxacin, erythromycin, and tetracycline, a perfect correlation between antimicrobial phenotypes and genotypes was found. Overall, our data suggest that WGS analysis is a powerful tool for identifying resistance determinants in *Campylobacter* and can disclose the full genetic elements associated with resistance, including antimicrobial compounds not tested routinely in antimicrobial susceptibility testing.

KEYWORDS *Campylobacter*, multidrug resistance, whole-genome sequencing, aminoglycoside resistance, aminoglycoside-modifying enzymes

Campylobacter jejuni and *Campylobacter coli* are the leading bacterial causes of human gastroenteritis worldwide (1). These foodborne pathogens are usually transmitted via raw or undercooked meat (poultry and pork) (2). Antimicrobial therapy should not be used routinely, as the infection is often self-limiting. If needed, a macrolide can be used as first-line treatment (3). Alternative antibiotics, such as fluoroquinolones, can be prescribed based on *in vitro* susceptibility results. Complications, such as septicemia, can occur sporadically and may require the use of gentamicin, a highly effective aminoglycoside (4).

Antimicrobial resistance is increasing at an alarming rate in *Campylobacter*. In France, resistance rates of more than 50% are currently observed for tetracyclines and fluoroquinolones and can be even higher in other parts of Europe, such as Portugal (5, 6). In contrast,

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resistance to aminopenicillins and macrolides is stable in France (data from the French National Reference Center for *Campylobacter*) (5), while the rate of *C. coli* strains resistant to macrolides has reached 50% in Portugal (6). It is noteworthy that resistance rates for *C. coli* are usually higher than those for *C. jejuni* (7, 8). Generally speaking, gentamicin resistance is very rare in human isolates but is emerging in some countries, especially in Asia, and may be due to an abusive use of antibiotics in farm animals (8–11).

Acquired antimicrobial resistance mechanisms in *Campylobacter* have been described widely (12). Various mechanisms can coexist in the same bacterial cell for a specific class of antibiotics and can have an impact on the MIC levels. Regarding tetracyclines, fluoroquinolones, macrolides, and aminopenicillins, mechanisms are well documented for *Campylobacter* and are mostly due to *tet*(*O*), a Thr-86-Ile mutation encoded within the quinolone resistance-determining region (QRDR) of *gyrA*, an A2075G mutation in the 23S rRNA gene, and a G57T transversion upstream of the promoter region of bla_{OXA-61}, respectively (13–15).

Enzymatic modification is the most prevalent and important mechanism of aminoglycoside resistance in *Campylobacter* spp., but new enzymes are frequently described (16–20). Aminoglycoside-modifying enzymes are divided into the following three distinct families, based on the reactions that they catalyze: aminoglycoside acetyltransferases (AAC), aminoglycoside phosphotransferases (APH), and aminoglycoside nucleotidyltransferases (ANT) (21). The main enzyme classes described for *Campylobacter* spp. are represented by APH(3′), APH(2″), ANT(6), ANT(9), and AAC(3). Regarding APH(3′), APH(3′)-IIIa is the most prevalent phosphotransferase in *Campylobacter* (21–24). APH(2″) usually confers resistance to gentamicin, with diverse phenotypes (25). APH(2″) enzymes are widely distributed in enterococci and staphylococci and are part of bifunctional enzymes associated with AAC(6′) in those genera (26, 27). They are generally found as monofunctional enzymes in *Campylobacter* but can also be found as bifunctional enzymes (16). Eight types of APH(2″) have been described so far (variants not included), seven of which have already been identified in *Campylobacter* (16, 17, 19, 21). The enzymes ANT(6) and ANT(9) confer resistance to streptomycin and spectinomycin, respectively, and can be encoded by different genes (18, 21, 22).

The present work aimed to identify putative genetic determinants associated with resistance to several antibiotics in 12 multidrug-resistant *Campylobacter* strains by using whole-genome sequencing (WGS).

MATERIALS AND METHODS

***Campylobacter* strains, culture, and susceptibility testing.** Twelve *Campylobacter* strains (eight *C. coli* and four *C. jejuni* strains) isolated from human fecal samples, except for one *C. coli* pork meat isolate, were selected for their *in vitro* resistance to gentamicin. Eight strains came from the French National Reference Center of *Campylobacter* and *Helicobacter* (CNRCH; Bordeaux, France) (https://www.cnrch.fr/), and four *C. coli* strains were from the Portuguese National Reference Laboratory of Gastrointestinal Infections (Lisbon, Portugal) (Table 1). These 12 strains were isolated during the period of 2006 to 2014 and represent the entire collection of gentamicin-resistant *Campylobacter* spp. isolated during this period. Strains were stored at −80°C in brucella broth supplemented with 25% glycerol. *Campylobacter* strains were grown on tryptic soy agar supplemented with 5% horse blood (TSH; bioMérieux, Marcy l'Etoile, France) under microaerobic conditions (6% O_2, 10% CO_2, 85% N_2, and 5% H_2) at 37°C. Antimicrobial susceptibility testing was performed by the disk diffusion method according to European Committee for Antimicrobial and Susceptibility Testing (EUCAST) recommendations, using Mueller-Hinton agar supplemented with 5% defibrinated horse blood (MH-F) and 20 mg/liter of β-NAD (Sigma-Aldrich, Merck, Darmstadt, Germany) under the same atmospheric and temperature conditions, as previously described (28). For ciprofloxacin, erythromycin, and tetracycline, the EUCAST breakpoints (V8.0; January 2018) (http://www.eucast.org/clinical_breakpoints/) were used to classify strains as susceptible or resistant, while for the other molecules the cutoffs of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (V.1.0; March 2017) were considered (http://www.sfm-microbiologie.org/page/page/showpage/page_id/90.html). The MIC of gentamicin for each isolate was determined under the same conditions, using Etest strips (bioMérieux) and the CA-SFM cutoff. The reference strain *C. jejuni* ATCC 33560 was used as a quality control strain, according to EUCAST recommendations.

Whole-genome sequencing and analysis. Briefly, high-quality genomic DNA samples from pure bacterial cultures were used to prepare Nextera XT Illumina libraries that were sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, CA) by use of a v.2 (300 cycles with 2 × 150-nucleotide [nt] reads or 500 cycles with 2 × 250-nt reads) kit according to the manufacturer's instructions. The FastQC v0.11.3

TABLE 1 Summary of antimicrobial resistance profiles and whole-genome sequencing data for *Campylobacter* strains included in this study[a]

Isolate (yr of isolation/strain-country)	Species	Resistance profile		Gentamicin MIC (mg/liter)	Genomic data					Size (~kb) of large plasmid carrying antibiotic resistance determinants (%GC)
		Aminoglycosides[b]	Other antibiotics[c]		Genome size (bp)	% GC	No. of contigs	No. of CDS	SRA accession no.	
2006/550-Fr	*C. coli*	Kan Tob Gen Spc Stp	Cip Tet Amp	>256	1,753,999	31.3	28	1,832	SRP090006	
2006/1293-Fr	*C. jejuni*	Kan Tob Gen Spc Stp	Cip Tet	>256	1,703,186	30.6	22	1,763	SRP090015	60 (32.4)
2014/1809-Pt	*C. coli*	Kan Tob Gen Spc Stp	Cip Tet Amp Ery	48	1,733,603	31.4	30	1,808	SRP090013	
226199F-Pt	*C. coli*	Kan Tob Gen Spc Stp	Cip Tet Ery	8	1,848,415	31.1	54	1,943	SRP090019	
2006/490H-Fr	*C. coli*	Kan Tob Gen Spc Stp	Cip Tet Amp	64	1,654,914	31.5	30	1,702	SRP090007	
2010/378-Fr	*C. coli*	Kan Tob Gen Spc	Cip Tet Amp Ery	96	1,804,469	31.0	60	1,874	SRP090010	53 (29.7)
2014/105H-Fr	*C. jejuni*	Kan Tob Gen Spc	Cip Tet Amp	32	1,815,943	30.1	71	1,925	SRP090018	
2012/873-Pt	*C. coli*	Kan Tob Gen Spc	Cip Tet Amp Ery	64	1,780,367	31.1	31	1,840	SRP090012	
2007/817-Fr	*C. coli*	Kan Tob Gen Stp	Cip Tet Amp Ery	>256	1,722,896	31.3	93	1,785	SRP090008	
2009/2424-Fr	*C. jejuni*	Kan Tob Gen Stp	Cip Tet	48	1,803,379	30.4	35	1,924	SRP090016	60 (32.5)
2012/2640-Fr	*C. jejuni*	Kan Tob Gen	Cip Tet	>256	1,672,987	30.4	49	1,732	SRP090017	52 (29.3)
2010/223-Pt	*C. coli*	Kan Tob Gen	Cip Tet Amp Ery	64	1,853,178	31.0	78	1,940	SRP090011	

[a]F, strain isolated from food (pork meat); Kan, kanamycin; Tob, tobramycin; Gen, gentamicin; Spc, spectinomycin; Stp, streptomycin; Cip, ciprofloxacin; Tet, tetracycline; Amp, ampicillin; Ery, erythromycin; CDS, coding sequences; Fr, France; Pt, Portugal.

[b]Using CA-SFM breakpoints for ampicillin, gentamicin, kanamycin, tobramycin, spectinomycin, and streptomycin.

[c]Using EUCAST breakpoints for ciprofloxacin, tetracycline, and erythromycin.

(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and FASTX v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/) software tools were applied to evaluate and improve, respectively, the quality of the raw read sequence data. Draft genomes were *de novo* assembled using SPAdes v3.7.1. with the default settings, except for the "careful" option for mismatch correction (29). The existence of putative plasmids was inspected by checking contigs of >1,000 bp with a high depth of coverage and evidence of circularity, while the presence of known plasmids was examined by reference-based mapping using Bowtie2, version 2.1.0 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), against a list of plasmids already described for *Campylobacter* spp.

Antibiotic resistance gene identification. The Rapid Annotation using Subsystem Technology (RAST) server was used for annotation of the whole genome in order to identify genes by functional variant recognition. Resistance genes corresponding to major antibiotic families in *Campylobacter* were sought in the annotated genome and analyzed (nucleotide and protein sequences) using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences carrying similar putative resistance genes from the sequenced genomes in this study were then aligned by use of Multalin (http://multalin.toulouse.inra.fr/multalin/) with each other and with resistance genes known from previous studies in order to assess their degree of homology and the possible mutations present. MEGA 7 (30) was used to align the protein coding sequences by using the ClustalW algorithm and to generate phylogenies at the protein level. Trees were constructed using the neighbor-joining method (31) with the Dayhoff matrix model to calculate evolutionary amino acid distances (1,000 replicates).

Accession number(s). Raw sequence reads for the 12 *Campylobacter* strains subjected to WGS have been deposited in the Sequence Read Archive (SRA) under accession numbers SRP090006 to SRP090008, SRP090010 to SRP090013, and SRP090015 to SRP090019. The novel sequences of the resistance-associated genes identified in the present study were submitted to GenBank and are available under the following accession numbers: KX931102 [for *aph*(2″)-*Ii*$_1$_Cje2640], KX931103 [for *aph*(2″)-*Ii*$_1$_Cje1293], KX931104 [for *aph*(2″)-*Ii*$_1$_Cco817], KX931105 [for *ant*(6)-*Ig*_Cco550], KX931106 [for *ant*(6)-*Ig*_Cco490H], KX931107 [for *ant*(6)-*Ig*_Cco226199], KX931108 [for *lnu*(G)_Cje105H], KX931109 [for *lnu*(G)_Cco378], KX931110 [for *aph*(2″)-*Ii*$_2$_Cco873], and KX931111 [for *aph*(2″)-*Ii*$_2$_Cco223].

RESULTS AND DISCUSSION

Summary of antimicrobial resistance profiles and WGS data. The 12 *Campylobacter* strains were selected based on their multidrug resistance profiles and, in particular, their resistance to gentamicin. Gentamicin MICs varied from 8 mg/liter to >256 mg/liter (Table 1). In addition, all strains were resistant to kanamycin and tobramycin, eight strains were resistant to spectinomycin, and seven were resistant to streptomycin. Regarding the other antimicrobials, all strains were resistant to ciprofloxacin and tetracycline, eight were resistant to ampicillin (seven *C. coli* and one *C. jejuni* strain), and six *C. coli* strains were resistant to erythromycin. Only three *C. jejuni* strains remained susceptible to both erythromycin and ampicillin. Draft genomes for these isolates were estimated to vary between 1.66 and 1.86 Mb. The mean coverage depth (per base) varied from 122 to 512 times, and the overall %GC was ~31%. Large plasmids (>50 kb) carrying antibiotic resistance determinants were detected in at least four strains (three *C. jejuni* and one *C. coli* strain) (Table 1).

Identification of aminoglycoside-associated resistance genes. Two types of aminoglycoside acetyltransferase genes were found in the genomes of the 12 gentamicin-resistant *Campylobacter* strains studied: genes for *N*3′-aminoglycoside acetyltransferases [also called *N*-acetyltransferases, acetyltransferases, or AAC(3)] (1 to 3 per genome) and genes for GCN5-related *N*-acetyltransferases (*gnat*) (3 to 7 per genome) (see Table S1 in the supplemental material). There was no association between the number of aminoglycoside acetyltransferase genes and the MIC level for gentamicin. Also, it is unknown if all these alleles are functional.

The *aac*(3) genes, usually associated with resistance to gentamicin and tobramycin, among others (21, 22), were highly diverse at both the nucleotide and amino acid levels, generally showing low homology with each other and with known *aac*(3) genes previously described for *Campylobacter* (21; data not shown).

The GNAT superfamily encompasses more than 100,000 proteins among eukaryotic and prokaryotic organisms and is divided into different groups according to specific substrates (aminoglycosides, peptidoglycans, histones, etc.) (32). Three of the multiple *gnat* genes identified in the 12 genomes from the present study (Table S1) (from *C. coli* 2006/550, *C. coli* 2010/378, and *C. jejuni* 2014/105H) were 100% identical at the protein level to the *aac* gene described for the *C. jejuni* plasmid pCG8245 (23) (Table S2), although in our strains they were located on the chromosome. Nirdnoy et al. showed

TABLE 2 Summary of resistance phenotypes and genotypes identified in 12 *Campylobacter* strains[a]

Antibiotic class	Drug tested	No. of resistant isolates (n = 12)	Resistance gene(s) or mutation(s) associated with resistance phenotype	Correlation between phenotype and genotype (% [no. of strains with correlation/total no. of strains])
Aminoglycosides	Gen	12	*aph*(2″)-*IIIa*, *aph*(2″)-*If*, *aph*(2″)-*Ii*$_1$, *aph*(2″)-*Ii*$_2$, *apmA*	100 (12/12)
	Kan	12	*aph*(2″)-*IIIa*, *aph*(2″)-*If*, *aph*(2″)-*Ii*$_1$, *aph*(2″)-*Ii*$_2$, *aph*(3′)-*IIIa*, *aac*	100 (12/12)
	Tob	12	*aph*(2″)-*IIIa*, *aph*(2″)-*If*, *aph*(2″)-*Ii*$_1$, *aph*(2″)-*Ii*$_2$	100 (12/12)
	Stp	7	*ant*(6)-*If*, *ant*(6)-*Ig*	100 (7/7)
	Spc	8	*ant*(9)-*Ic*, *spw**, 16S rRNA gene C1192T	100 (8/8)
Tetracyclines	Tet	12	*tet*(O)	100 (12/12)
Quinolones	Cip	12	*gyrA* Thr-86-Ile	100 (12/12)
β-Lactams	Amp	8	bla_{OXA-61} G57T	100 (8/8)
Macrolides	Ery	6	23S rRNA gene A2075G	100 (6/6)

[a]Gen, gentamicin; Kan, kanamycin; Tob, tobramycin; Stp, streptomycin; Spc, spectinomycin; Tet, tetracycline; Cip, ciprofloxacin; Amp, ampicillin; Ery, erythromycin; *, truncated gene.

that the protein encoded by this *aac* gene is functional and confers kanamycin resistance (23).

Interestingly, an acetyltransferase (present in *C. coli* strain 226199F) first annotated via RAST as a chloramphenicol acetyltransferase was then identified as an ApmA aminocyclitol acetyltransferase via BLASTP. The gene revealed 100% identity with *apmA*, first described in 2011 for methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from bovine and porcine samples and shown to confer resistance to apramycin in *S. aureus* (33) (Tables S1 and S2). Apramycin is an antimicrobial drug of the aminoglycoside family used only in veterinary medicine against Gram-negative bacterial infections, such as *Salmonella* or *Escherichia coli* infections, mainly in pigs but also in other farm animals (33, 34). The ApmA enzyme is usually associated with a low level of gentamicin hydrolysis, which is perfectly in line with the low gentamicin MIC found for this strain (8 mg/liter) (Table 1). It is particularly interesting that *C. coli* strain 226199F was isolated from pork meat in Portugal. It was suggested that *apmA* can be mobilized by horizontal gene transfer between MRSA and methicillin-susceptible *S. aureus* (MSSA) strains isolated from pigs in Portugal (35). Therefore, it is highly likely that this mechanism contributes to the dissemination of this gene among different species. This is the first report identifying *apmA* in *Campylobacter*.

Concerning aminoglycoside phosphotransferases, numerous *aph*(3′) genes, associated with kanamycin resistance, were found in 9 of the 12 sequenced strains (Table 2; Tables S1 and S2). Alignment of the protein sequences showed that they were all encoded by the *aph*(3′)-*IIIa* (also called *aphA-3*) gene, which is the most frequently found *aph*(3′) gene in *Campylobacter* spp. (21, 22). For two strains (*C. coli* 226199F and *C. jejuni* 2009/2424), this gene was located within the previously described *aadE-sat4-aphA-3* chromosomal resistance cluster (Table S2) (36–38).

The *aph*(2″) genes were found in all but one gentamicin-resistant strain (*C. coli* 226199F), and they displayed high genetic diversity (Fig. 1; Table 1; Tables S1 and S2). Four different monofunctional *aph*(2″) genes were identified in these 11 genomes. The *aph*(2″)-*IIIa* and *aph*(2″)-*If* genes were each found in three genomes (Table S1), and two new *aph*(2″) genes that were very similar to each other (96% amino acid identity) were identified in five different strains (*C. jejuni* 2006/1293, *C. coli* 2012/873, *C. coli* 2007/817, *C. jejuni* 2012/2640, and *C. coli* 2010/223) (Tables S1 and S2). Phylogenetic analysis of APH(2″) followed that carried out by Zhao et al. (16) upon discovery of APH(2″)-Ih and took into account the two new variants of APH(2″) identified in this work. These new variants were therefore designated APH(2″)-Ii$_1$ and APH(2″)-Ii$_2$. APH(2″)-Ii was phylogenetically close to APH(2″)-If and -Ih (80 to 83% identity and 90 to 92% homology) and was most likely derived from a common ancestor to be part of a new APH(2″) subfamily (Fig. 1). It is noteworthy that the *aph*(2″)-*Ii*$_1$ gene was systematically found as part of a new cluster that we named *gnat/aph*(2″)-*Ii*$_1$/*aph*(3′)-*IIIa* (Table S2), located on the

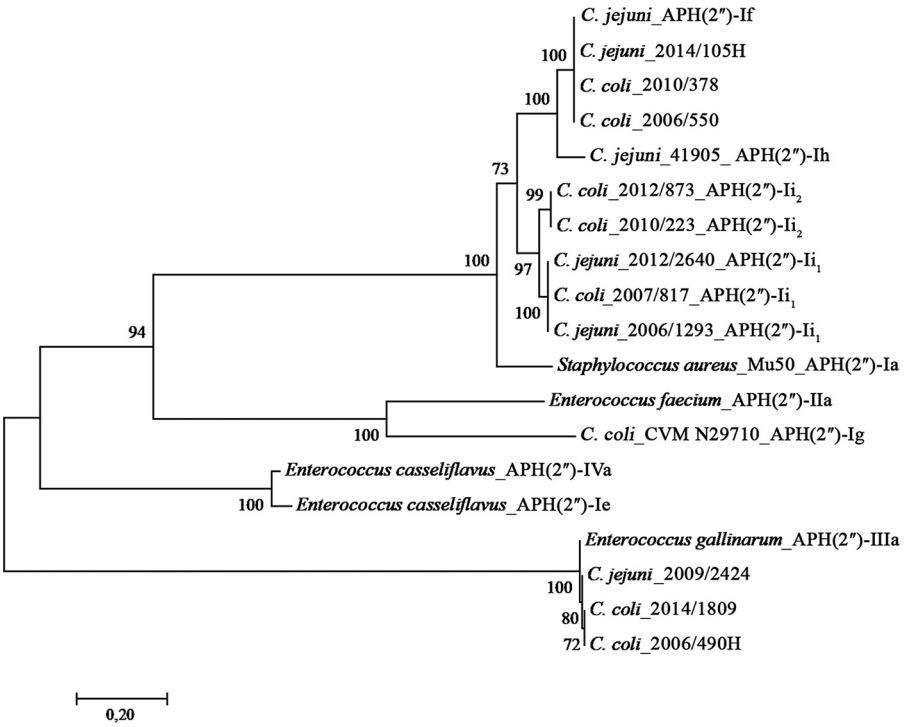

FIG 1 Phylogenetic relationships between APH(2″) aminoglycoside phosphotransferases. GenBank accession numbers for the enzymes studied are as follows: *Staphylococcus aureus* Mu50 APH(2″)-Ia, BAB47534.1; *Enterococcus faecium* APH(2″)-IIa, AF207840.1; *Enterococcus gallinarum* APH(2″)-IIIa, U51479.1; *Enterococcus casseliflavus* APH(2″)-IVa, AF016483.1; *Enterococcus casseliflavus* APH(2″)-Ie, AY939911.1; *Campylobacter jejuni* APH(2″)-If, AAW34150.1; *Campylobacter coli* CVM N29710 APH(2″)-Ig, AGV10818.1; and *Campylobacter jejuni* 41905 APH(2″)-Ih, KF652096.1. The tree was constructed with the protein sequences by using the neighbor-joining method with the Dayhoff matrix method to calculate evolutionary distances (1,000 replicates).

chromosome (in *C. jejuni* 2012/2640 and *C. jejuni* 2006/1293) or at a plasmid site (in *C. coli* 2007/817). By focusing on the gentamicin resistance level, we found that the three strains harboring *aph*(2″)-*Ii*$_1$ (*C. jejuni* 2006/1293, *C. coli* 2007/817, and *C. jejuni* 2012/2640) all had high-level resistance (MIC $>$ 256 mg/liter), which was not the case for the two strains with *aph*(2″)-*Ii*$_2$ (MIC = 64 mg/liter). This result suggests that the *aph*(2″)-*Ii*$_1$ variant may confer a higher resistance level to gentamicin than that with the *aph*(2″)-*Ii*$_2$ variant, although the limited number of strains hampers a more definitive conclusion.

There were three strains harboring the *aph*(2″)-*If* gene. Two of them had an intermediate level of gentamicin resistance (*C. jejuni* 2014/105H [MIC = 32 mg/liter] and *C. coli* 2010/378 [MIC = 96 mg/liter]), which corresponds to the phenotype associated with this gene, as previously described (19). However, the *C. coli* 2006/550 strain presented a higher level of resistance to gentamicin (MIC $>$ 256 mg/liter). Therefore, the level of resistance associated with the presence of *aph*(2″)-*If* may depend on the bacterial species and remains to be clarified. Concerning aminoglycoside nucleotidyltransferases, 7 of the 12 strains had a putative *ant*(*6*) gene, and 4 of them (*C. coli* 2014/1809, *C. coli* 226199F, *C. coli* 2006/490H, and *C. jejuni* 2009/2424) had two or three different copies of this gene (Table S1). All of these strains were resistant to streptomycin (Table 1). In most strains (6/7 strains), the previously described *aadE* gene (ORF2; *C. jejuni* plasmid pCG8245) was found (23). The *ant*(*6*) gene was named *ant*(*6*)-*If* in the present study to distinguish it from others identified previously. It seemed to be related more closely to *ant*(*6*)-*Ib*, described and identified for a genomic island of a streptomycin-resistant *Campylobacter fetus* subsp. *fetus* strain (18) (Fig. 2).

Three strains (*C. jejuni* 2009/2424, *C. coli* 2006/490H, and *C. coli* 226199F) harbored an *ant*(*6*) gene identical to the *aadE* gene described for the *aadE-sat4-aphA-3* resistance cluster (ORF23; *C. jejuni* plasmid pCG8245) (23) and closely related (86% identity) to

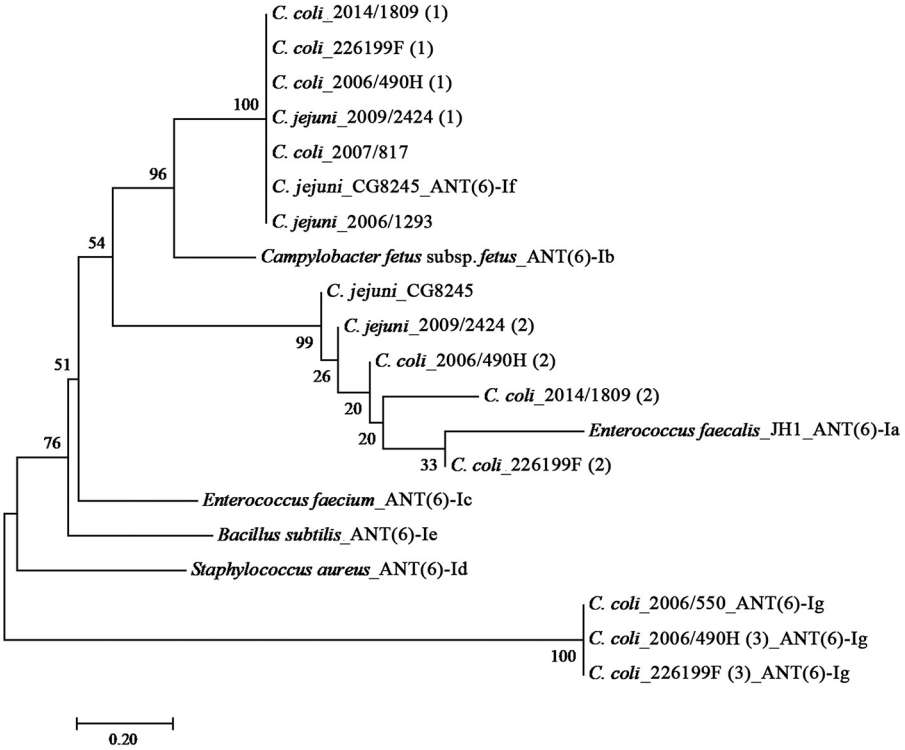

FIG 2 Phylogenetic relationships between ANT(6) nucleotidyltransferases. GenBank accession numbers for the enzymes studied are as follows: *Enterococcus faecalis* JH1 ANT(6)-Ia, ZP_05574545.1; *Campylobacter fetus* subsp. *fetus* ANT(6)-Ib, CBH51824.1; *E. faecium* ANT(6)-Ic, AAR10415.1; *Staphylococcus aureus* ANT(6)-Id, P12055.1; *Bacillus subtilis* ANT(6)-Ie, M26879.1; and *Campylobacter jejuni* CG8245 ANT(6)-If (encoded by *aadE* [ORF2] [23]), AAW34139.1. The tree was constructed with the protein sequences by using the neighbor-joining method with the Dayhoff matrix method to calculate evolutionary distances (1,000 replicates).

ant(*6*)-*Ia*, which is highly disseminated in Gram-positive bacteria (Tables S1 and S2). A fourth putative *ant*(*6*) gene was identified in three strains (*C. coli* 2006/550, *C. coli* 2006/490H, and *C. coli* 226199F) (Tables S1 and S2). This streptomycin-associated resistance gene was recently described and characterized for *C. coli* and named *ant*-like gene A (39). Functional studies confirmed that this gene induces streptomycin resistance in both *Campylobacter* and *Escherichia coli* (39). We propose naming it *ant*(*6*)-*Ig* to follow the previously used nomenclature for other *ant*(*6*) genes that have already been described.

Seven of the eight spectinomycin-resistant strains also contained *ant*(*9*) genes (Fig. 3). Six strains (*C. coli* 2006/550, *C. jejuni* 2006/1293, *C. coli* 226199F, *C. coli* 2010/378, *C. jejuni* 2014/105H, and *C. coli* 2006/490H) shared the same gene, first characterized by Nirdnoy et al. (23) and associated with resistance to spectinomycin (Table 2; Tables S1 and S2). Its closest homologue is *ant*(*9*)-*Ia* (67% amino acid homology). In the present study, it was named *ant*(*9*)-*Ic*, although its functionality was not proven. One strain (*C. coli* 2014/1809) harbored a copy of a recently described *ant*(*9*) gene (40) (Fig. 3). This gene was first identified in the genetic environment of MRSA (41, 42) and is an *spw* gene which encodes two conserved functional domains: a kanamycin nucleotidyltransferase domain in the N-terminal region (which was truncated in *C. coli* 2014/1809) and a domain of unknown function in the C-terminal part. The last domain may be responsible for the enzymatic inactivation of spectinomycin, but this needs to be confirmed by functional studies. This is the first report to identify the *spw* gene in *Campylobacter*.

Summaries of the putative aminoglycoside-associated resistance genes and their corresponding annotations are provided in Table 2 and in Tables S1 and S2. The putative aminoglycoside resistance genes found in the 12 studied strains were organized in several different resistance clusters. These results are shown in Fig. S1 to S3.

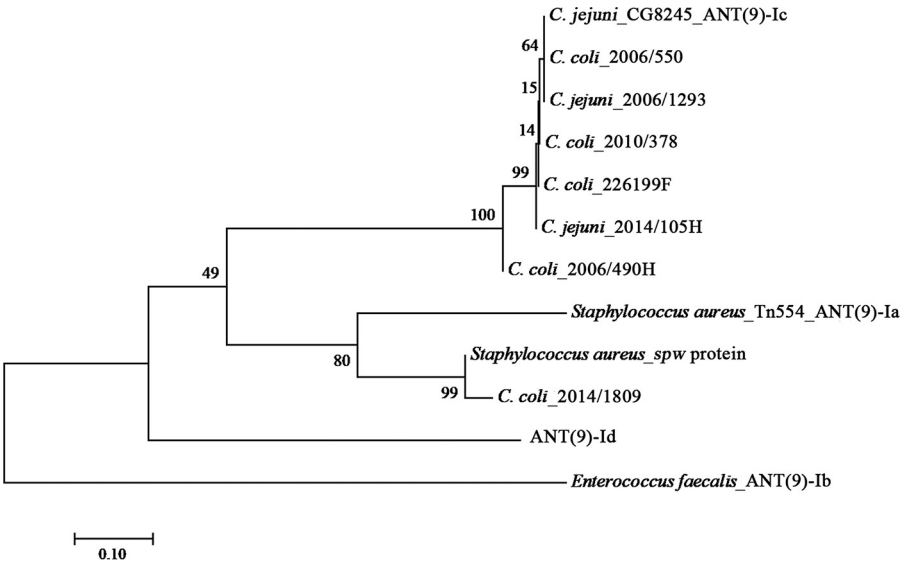

FIG 3 Phylogenetic relationships between ANT(9) nucleotidyltransferases. GenBank accession numbers for the enzymes studied are as follows: *S. aureus* Tn*554* ANT(9)-Ia, X02588.1; *Enterococcus faecalis* ANT(9)-Ib, M69221.1; *Campylobacter jejuni* CG8245 ANT(9)-Ic, AAW34145.1 (23); ANT(9)-Id, JN625765.1; and *S. aureus spw*-encoded protein, AFU35063 (40). The tree was constructed with the protein sequences by using the neighbor-joining method with the Dayhoff matrix method to calculate evolutionary distances (1,000 replicates).

Other antibiotic resistance determinants among sequenced strains. We found the *tet*(O) gene in all 12 *Campylobacter* strains, which were all resistant to tetracyclines (Table 2). For eight strains, *tet*(O) was located on the chromosome, and for three strains (*C. coli* 2010/378, *C. jejuni* 2012/2640, and *C. jejuni* 2009/2424), *tet*(O) was located on a resistance plasmid. The *C. jejuni* 2006/1293 strain had *tet*(O) on both the chromosome and the plasmid.

The usual *gyrA* mutation in the QRDR leading to a Thr-86-Ile substitution was found in all strains tested, supporting the fluoroquinolone-resistant phenotype (Table 2). No other, less common mutations (Ala-70-Thr and Asp-90-Asn) associated with moderate levels of resistance (43) were found.

The bla_{OXA-61} gene, encoding the most frequently found β-lactamase among *Campylobacter* strains, was found in 10 strains, with eight of them, all resistant to aminopenicillins (Table 2), harboring the G57T transversion upstream of the promoter region allowing expression of the gene, as previously described (14). Another protein, annotated as a metallo-β-lactamase, was found in three different *C. jejuni* strains, two of which were ampicillin susceptible (2006/1293 and 2009/2424) and one of which was ampicillin resistant (2014/105H). This enzyme was actually characterized as a glyoxalase II enzyme and was not associated with resistance to β-lactams, showing no ortholog in *C. coli*, as previously demonstrated (44).

The A2075G mutation in the 23S rRNA gene was present in all erythromycin-resistant strains and was absent in the susceptible ones (Table 2). The *erm*(*B*) gene, encoding an RNA methylase and described recently for macrolide-resistant *Campylobacter* (45), was not found.

Other antibiotic resistance determinants for *Campylobacter* identified in the present study. Two strains (*C. jejuni* 2014/105H and *C. coli* 2010/378) harbored a gene that was annotated as either *ant*(*2″*) or a lincosamide nucleotidyltransferase gene (*lnu*). Alignment with the *ant*(*2*)-*Ia* sequence, however, showed very limited amino acid homology, whereas alignment with gene sequences coding for lincosamide resistance enzymes showed 60% identity and 76% homology to *lnu*(C) of *Streptococcus agalactiae* (GenBank accession number AY928180.1) (46) and 62% identity and 75% homology to *lnu*(D) of *Streptococcus uberis* (GenBank accession number EF452177.1) (47). The *lnu*(C)

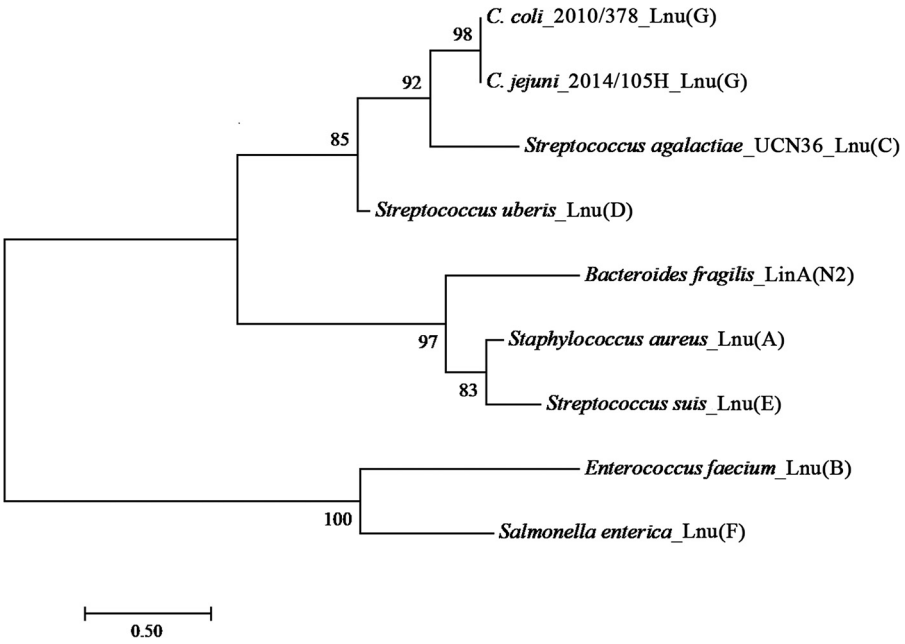

FIG 4 Phylogenetic relationships between lincosamide *O*-nucleotidyltransferases. GenBank accession numbers for the enzymes studied are as follows: *Staphylococcus aureus* Lnu(A), NC_019146.1; *Bacteroides fragilis* $LinA_{N2}$, AAF74724; *Enterococcus faecium* Lnu(B) or LinB, AF110130.1; *Streptococcus agalactiae* UCN36 Lnu(C), AY928180.1; *Streptococcus uberis* Lnu(D), EF452177.1; *Streptococcus suis* Lnu(E), KF287643.1; and *Salmonella enterica* Lnu(F), EU118119.1. Lnu(G) sequences were from this study (found in *Campylobacter coli* 2010/378 and *Campylobacter jejuni* 2014/105H). The tree was constructed with the protein sequences by using the neighbor-joining method with the Dayhoff matrix method to calculate evolutionary distances (1,000 replicates).

gene had already been described for *Campylobacter* (24), but *lnu*(D) had not. The *in vitro* susceptibility profiles of both strains were verified, and strain *C. coli* 2010/378 was found to be resistant not only to lincomycin but also to erythromycin and clindamycin (A2075G mutation in the 23S rRNA gene), while *C. jejuni* strain 2014/105H was resistant to lincomycin only (data not shown). Therefore, the gene may be a new lincosamide nucleotidyltransferase gene that we propose to name *lnu*(G), but this needs to be confirmed by functional studies. Construction of the phylogenetic tree of *O*-lincosamide nucleotidyltransferases followed that for the one established by Petinaki et al. (47) and integrated this putative new resistance gene which is phylogenetically closer to *lnu*(C) and *lnu*(D) (Fig. 4).

In one of the eight spectinomycin-resistant strains (*C. coli* 2012/873), no *ant*(*9*) or *aph*(*9*) genes were present. In addition, no mutations were found in the *rpsE* gene, encoding the S5 protein of the 30S bacterial ribosome subunit, previously described for spectinomycin-resistant strains from several species, namely, *E. coli*, *Pasteurella multocida*, and *Neisseria gonorrhoeae* (48–50). Analysis of the nucleotide sequence of the 16S rRNA gene of the small ribosomal subunit, however, permitted us to identify a C1273T transition that may be associated with spectinomycin resistance in this *C. coli* strain. Indeed, this mutation corresponds to the C1192T substitution (*E. coli* numbering) of the upper region of helix 34 identified in many bacterial species, which confers a high level of resistance to spectinomycin (51–53). Further investigations will be necessary to confirm the association of the C1273T transition in the 16S rRNA gene with spectinomycin resistance in *C. coli*, which has not previously been described.

Finally, two *C. coli* strains (2006/490H and 2006/550) harbor a gene encoding a putative hygromycin B phosphotransferase, known to confer resistance to hygromycin. The alignment of these two sequences with those of known hygromycin resistance genes described for other bacterial species, such as *aph*(*4*)-*Ia* in *E. coli* and *aph*(*7″*)-*Ia* in *Streptomyces hygroscopicus* (54, 55), showed relatively low homology (57% and 36%

amino acid homology, respectively). The homology was 100%, however, with the HPH protein previously described for *C. jejuni* (23) to be associated with hygromycin, despite a truncated N-terminal domain in *C. coli* 2006/550 (data not shown).

Conclusions. Seventeen putative aminoglycoside-associated resistance genes or mutations were identified in the 12 gentamicin-resistant *Campylobacter* strains included in this study, including putative new aminoglycoside-modifying enzymes as well as a probable new lincomycin resistance gene. These genes were randomly distributed either on the bacterial chromosome or on plasmids. Although the number of sequenced isolates is small and genetic or functional studies are needed to confirm our WGS findings, our data suggest that WGS might be a useful tool for investigating antibiotic resistance determinants in *C. jejuni* and *C. coli*.

A perfect correlation between antimicrobial phenotypes and genotypes was found regarding the antibiotics ciprofloxacin, tetracycline, ampicillin, and erythromycin. Concerning aminoglycosides, the correlation between resistance phenotypes and genotypes was more complex to determine, taking into account the presence of several known or putative resistance genes in certain strains. Nevertheless, the results suggest that high-level gentamicin resistance is likely associated with the presence of *aph*(2″) genes, with a new variant, *aph*(2″)-*Ii*, described here. For one strain isolated from food, with a low level of resistance, the presence of an *apmA* gene coding for an aminocyclitol acetyltransferase likely explains the phenotype of this strain that lacks an *aph*(2″) gene.

Regarding spectinomycin, a correlation between phenotype and genotype was found, with new putative mechanisms being suggested, i.e., the presence of the *spw* gene in one *C. coli* strain and the 16S rRNA gene C1273T mutation found in another *C. coli* strain. The presence of *apmA* and *spw* genes as well as the 16S rRNA gene C1273T mutation in *Campylobacter* spp. reported here suggests that genetic exchange via horizontal gene transfer between *Campylobacter* and other bacterial genera is possible and may contribute to the emergence of resistance. Concerning the other aminoglycosides tested (kanamycin, tobramycin, and streptomycin), a perfect correlation was found between phenotype and genotype. The correlations between phenotype and genotype for all the antibiotics tested are summarized in Table 2.

Our results confirm those of previous WGS-based studies describing high levels of correlation between resistance genotypes and phenotypes (16, 17, 23, 24, 39). The large number of putative new resistance genes and antimicrobial resistance mechanisms described by analysis of only 12 *Campylobacter* genomes shows the large potential that this technology may bring to the field of antimicrobial resistance. However, studies to examine issues such as unknown resistance mechanisms, undetected resistance genes, and gene expression levels, as well as functional studies, remain to be performed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00390-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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