Emergence of erythromycin resistance methyltransferases in *Campylobacter coli* strains in France

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ABSTRACT

Antimicrobial resistance in Campylobacters is described worldwide. The emergence of multiresistant isolates, particularly among *C. coli*, is concerning. New resistance mechanisms appear frequently, and DNA-sequence-based methods such as whole genome sequencing (WGS) have become useful tools to monitor their emergence.

The genomes of 51 multiresistant French *Campylobacter* sp. clinical strains from 2018 to 2019 were analyzed to identify associated resistance mechanisms. Analyses of erythromycin-resistant strains revealed 23S ribosomal RNA mutations among most of them and two different methyltransferases in 4 strains: Erm(B) and a novel methyltransferase, here named Erm(N). The *erm(B)* gene was found in multidrug-resistant genomic islands, whereas *erm(N)* was inserted within CRISPR arrays of the CRISPR-cas9 operon. Moreover, using PCR screening in erythromycin-resistant strains from our collection, we showed that *erm(N)* was already present in 3 French clinical strains 2 years before its first report in 2018 in Quebec. Bacterial transformations confirmed that insertion of *erm(N)* into a CRISPR-cas9 operon can confer macrolide resistance. *Campylobacter* species are easily able to adapt to their environment and acquire new resistance mechanisms, and the emergence of methyltransferases in Campylobacters in France is a matter of concern in the coming years.
INTRODUCTION

Each year, Campylobacter species are responsible for 800,000 gastroenteritis infection cases in the USA (1) and 200,000 in the European Union (2). In France, while there are an estimated 68,000 foodborne infections each year (3), the number of campylobacteriosis cases is not clearly defined. Campylobacter sp. infection, mostly caused by the two main species C. jejuni and C. coli, typically occurs via the consumption of contaminated meat, especially chicken (4) (5). Symptoms include abdominal cramps, diarrhea and fever and can lead to a serious risk of complications at extreme ages of life in immunosuppressed, diabetic or cancer patients (6). Treatment of severe intestinal infections consists of the administration of a macrolide (e.g., azithromycin) or a fluoroquinolone (e.g., ciprofloxacin) (7). An increased rate of resistance to such molecules can be a problem when determining treatment options (8).

In fact, resistance to quinolone and tetracycline has drastically increased over the years, especially in Europe. Campylobacter resistance levels are evaluated all over Europe by EU Member State reference centers and are summarized in “The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food” of the ECDC (2). Globally, in 2018-2019, it was shown that C. coli displayed higher levels of resistance to important antimicrobials than C. jejuni. Resistance rates to ciprofloxacin/tetracycline were on average 59.3/47.2% and 65.2/71.3% for C. jejuni and C. coli, respectively. However, resistance to erythromycin, gentamicin and amoxicillin-clavulanic acid remained relatively low. The proportion of human erythromycin-resistant C. jejuni strains was 1.8% overall but was higher in C. coli (14.3%). Multidrug resistance (MDR)-type strain analyses for four antimicrobial classes (fluoroquinolones, macrolides, tetracyclines and aminoglycosides) also showed that the most common resistance profile in C. jejuni and C. coli was resistance to both ciprofloxacin and tetracycline, observed in 39.4% of C. jejuni strains and
51.9% of *C. coli* strains. More specifically, between 1986 and 2019 in France, resistance to ciprofloxacin and tetracycline reached a critical rate of 60% for *C. jejuni* and *C. coli* (9)

Erythromycin and ampicillin resistance rates remain however relatively low, at 0.4%-4% and 30% for both species, but should not be disregarded.

Very high levels of resistance to fluoroquinolones are mostly due to the presence of point mutations on GyrA at positions 86 and 90 (10). A rarer mechanism is also mentioned, implying modifications in the expression of the efflux pump CmeABC, described, for example, in China (11). Gentamicin and tetracycline resistance genes are mostly found within chromosomal genomic islands (12). Resistance to macrolides is relatively low in Europe. The ECDC report mentioned that erythromycin resistance in Europe is almost entirely acquired by 23S rRNA mutations (A2074G, A2074C and A2075G), but the recent discovery of the *erm(B)* gene is also a matter of concern. Widely distributed in gram-positive and gram-negative bacteria (13), this gene is more frequently observed in *C. coli* than in *C. jejuni*, as described in recent studies in China (14). In Europe, *erm(B)* has only been reported in *C. coli* from broilers and turkeys in Spain and from a broiler strain in Belgium among tetracycline and aminoglycoside resistance genes (15) (16), with erythromycin minimum inhibitory concentrations (MICs) over 512 mg/L. It is therefore recommended by the ECDC to analyze any highly resistant or MDR strains by molecular methods such as whole genome sequencing (WGS).

In the present study, the genomes of 51 clinically multiresistant *C. jejuni* and *C. coli* from the collection of the French National Reference Center for Campylobacters and Helicobacters (NRCCH) (www.cnrch.fr) between 2018 and 2019 were analyzed by WGS followed by computational genomics approaches to identify antimicrobial resistance markers. Among these resistance-associated genes, two methyltransferases were found: *erm(B)* and a rare *erm* gene, named *erm(N)* (N for new), described only once in Quebec in 2018 (17). PCR screening and complementary microbial...
approaches, such as bacterial transformations, allowed us to evaluate the importance of this resistance mechanism. This study is the first description of erythromycin resistance methyltransferases in Campylobacters isolated from French clinical cases. This emergence is a matter of concern in the coming years.
MATERIALS AND METHODS

Campylobacter strain datasets and antimicrobial susceptibility

Fifty-one strains were selected from the French National Reference Center for Campylobacters and Helicobacters (NRCCH) (Bordeaux, France). These strains were isolated from stools obtained from French clinical cases between 2018 and 2019 (Tab. 1) by clinical laboratories that participate in the NRCCH surveillance network and send their campylobacter isolates for epidemiological identification. The mean age and female percentage of this dataset were 37 and 41.2%, respectively. The collection comprised 9 erythromycin-resistant Campylobacter jejuni and 42 Campylobacter coli strains resistant to multiple antimicrobials (Tab. 1). All of the strains were recovered from frozen stocks (−80°C in in-house peptone +20% glycerol broth) on Columbia blood agar (CBA) plates with 5% sheep’s blood (Thermo Fisher Scientific, MA). Plate cultures were incubated at 37°C in jars using an Anoxomat microprocessor (Mart Microbiology, B.V. Lichtenvoorde, The Netherlands), which creates an atmosphere of 80–90% N₂, 5–10% CO₂, and 5–10% H₂. Single strains were plated onto CBA plates and used for species confirmation using MALDI-TOF mass spectrometry (MS) as previously described (18) and antimicrobial susceptibility testing (AST).

Antibiotic susceptibilities to ampicillin, ciprofloxacin, erythromycin, tetracycline and gentamycin were assessed based on the CASFM/EUCAST 2020 recommendations for Campylobacter sp. (https://www.sfm-microbiologie.org/2020/10/02/casfm-eucast-v1-2-octobre-2020/): Mueller–Hinton (MH) agar supplemented with 5% defibrinated horse blood (MH-F) and 20 mg/L β-NAD (bioMérieux, Marcy l’Etoile, France); inoculum: 0.5 McFarland standard; incubation for 24 h in a microaerobic environment as described above. MH-F plates were used and incubated at 37°C. For each strain, inhibition zone diameters were measured (Biorad, Marnes-La-Coquette, France) using an automatic system routinely applied in the laboratory for other bacterial species, SIRscan Auto (i2A,
Montpellier, France), as previously described (19). Following 24 hours of incubation, the point at which the zone of growth inhibition intersected the strip was read as the MIC in mg/L. The reference strain C. jejuni ATCC 33560 was used as a quality control strain, according to CASFM/EUCAST recommendations.

**DNA extraction, genome sequencing and assembly**

DNA was extracted from pure bacterial cultures using the MagNA Pure 6 DNA and Viral NA SV Kit, and DNA purification was performed from bacterial lysis on a MagNA Pure 96 System (Roche Applied Science, Manheim, Germany). Quantification and purity checks (260/280 and 260/230 ratios) were determined by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) before sequencing. Paired-end next-generation sequencing was performed on DNA samples using Illumina HiSeq 4000 technology (Integragen, Evry, France). Additionally, FastQC v0.11.8 (21) was used to run data quality tests. Genomic data were cleaned, and genomes were assembled using Sickle v1.33 (22) and SPAdes v3.10.1 (23), respectively. Sequences are available in the PubMLST and NCBI databases, and corresponding identifiers are listed in Tab. 1.

**Whole genome analyses**

Each analysis was performed either from raw fastq sequencing files or assembled fasta files using a Python homemade pipeline. First, species were confirmed using ANI calculation (average nucleotide identity) against 33 Campylobacter sp. references (listed in Fig. 1) with FastANI v1.1 (24). Multilocus sequence typing (MLST) was then performed using the sequence tag tool of PubMLST (25) and the conventional method established by Dingle et al. in 2001 (26). Additionally, whole genome SNP phylogeny was achieved by aligning all studied genomes to C. coli NTICC13 reference using Samtools v1.10 (27). Pairwise SNP comparisons were performed to obtain distances between...
all strains: 0% indicates absolute number of similar SNP and 100% indicates total divergence. Distances were displayed in a phylogenetic tree using MEGAX v10.1.7 (Fig. 1) (28).

Resistance genes were identified using Ariba v2.14.4 (29) based on the CARD v3.1.0 database (30) and an in-house *Campylobacter* resistance gene and mutation database. This last database has been manually constructed from multiple previously published studies (10) (12) (31). Phylogenetic trees of resistance genes were constructed from amino acid sequence alignment using Muscle v3.8.1551 (32), Fasttree v2.1.11 (33) and iTOL v5 (34). Finally, potential sources of contamination were estimated using STRUCTURE software v2.3.4 (35) for 15 host-segregating genes (36) and 259 SNPs (37) from *C. jejuni* and *C. coli* strains, respectively. *C. jejuni* markers allow us to discriminate isolates sampled from chickens, cattle and environmental reservoir hosts, whereas *C. coli* SNPs are able to differentiate chicken, cattle and pig isolates.

**PCR screening of identified methyltransferases**

PCR screening of *erm*(B) and *erm*(N) was performed on a new set of 141 and 30 erythromycin-resistant *C. coli* and *C. jejuni* strains, respectively, that were selected from our collection between 2016 and 2019 (mean age and female percentage of 42 and 40%, respectively). Primers for *erm*(B) were designed as follows: 5’-AAAGCCATGCCTGACATC-3’ (F) and 5’-CTTACCGCCATACCACAGA-3’ (R) with an expected PCR product size of 196 bp and primers for *erm*(N) 5’-TAGCGGTACAGACGAGTCC-3’ (F) and 5’-GATTCTGGCATGGGATCGG-3’ (R) with an expected size of 213 bp. Amplifications were performed using PCR with program #1 in Table S1 and analyzed on 2% agarose gels containing Midori Green Advance coloring (Nippon Genetics Europe, Düren, Germany).
Bacterial transformations

Bacterial transformations of a C. coli erythromycin-sensitive and CRISPR-cas9-positive strain (2019/0231H) with CRISPR-erm(N) products were performed in biphasic systems as described by Wang & Taylor in 1990 (38). Briefly, the 2019/0231H strain was initially grown on a CBA plate for 24 h (at 37°C in microaerobic conditions). Cells were selected (1 McF in 250 µL of MH broth, Thermo Fisher Scientific, MA) and grown on MH-F for 6 h using the same conditions. Incubations in biphasic systems were then performed for 5 h in 50 mL sterile polypropylene tubes containing 10 mL of MH-5% (sheep blood, Thermo Fisher Scientific, MA) + erythromycin (10 µg/mL), 1 mL of fresh cells in MH broth (1 McF) and 85 µL of PCR product (250 ng/µL). CRISPR-erm(N) regions were amplified using the primers 5’-CGCTTTTTGATATAGATAGTCATGG-3’ (F) and 5’-CAAGCGACAAATTCCAAATAA-3’ (R) with expected sizes of 2,403 bp (2019/0191) and 2,468 bp (2019/0051) and PCR program #2 in Table S1. Transformations were performed using 2 CRISPR-erm(N) PCR products obtained from C. coli strains 2019/0191 and 2019/0051. Finally, transformants were selected on MH-5% + erythromycin (10 µg/mL) for 48 to 72 h (at 37°C in microaerobic conditions). Growth controls were also realized using MH-F without erythromycin. Insertion of CRISPR-erm(N) PCR product into CRISPR-cas9-positive isolate 2019/0231H has been verified using PCR programs #1 and #2 and WGS.

Data availability

Assembled genomes are available under BioProject PRJNA717118. The initial 51 multiresistant strains under BioSamples SAMN18478564-SAMN18478614 (full accession list provided in Tab. 1) and additional erm-positive strains or those used for bacterial transformations were as follows: 2016/0429H: SAMN18478615; 2016/0940: SAMN18478616; 2016/2392: SAMN18478617; 2017/0180: SAMN18478618; 2018/1149: SAMN18478619; 2019/0231H/0051: SAMN18478620; 2019/0231H/0191: SAMN18478621; and 2019/0231H: SAMN18478624. 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: MZ015744 (erm(N)) and MZ015745/MZ015746/MZ015747 (erm(B)).
RESULTS

Genomic characterization of 51 selected multiresistant Campylobacter strains

The genomes of 51 Campylobacter sp. (42 C. coli and 9 C. jejuni) isolates were analyzed by WGS. C. coli genomes have a median genome length of 1.72 Mbp (range number of contigs from 23 to 304).

Concerning C. jejuni genomes, a median genome length of 1.76 Mbp was found (range number of contigs from 27 to 886). These results are consistent with other published C. coli and C. jejuni genomes, estimated to be ~1.7 Mbp in length (39) (40). Nearly all strains of C. coli belonged to the clonal complex CC-828 (88%, n=37), whereas the clonal complexes of the remaining 14% (n=6) have not been characterized yet (Tab. 1) (Fig. 1). Sequence types were more diverse, with ST-872, ST-860, and uncharacterized ST as the main sequence types encountered with 15, 4 and 5 strains, respectively.

By comparison, only 4 C. jejuni clonal complexes were identified: CC-353 (n=2), CC-52 (n=1) and CC-42 (n=1). Various sequence types were also identified: ST-6, ST-2066, ST-2274, ST-2328, ST-6461, ST-6532 and ST-7010. Source attribution of C. coli strains showed that chickens were estimated to be the main reservoir at 83% (n=35), followed by pigs at 17% (n=7). Similarly, a majority of C. jejuni strains were attributed to the chicken population, with 56% of all attributions (n=5), while 33% were attributed to the environment (n=3) and 11% to cattle (n=1). These results are in line with our previous publications (37) (41) showing that chickens are the main source of C. jejuni and C. coli campylobacteriosis in France.

Identification of antimicrobial resistance markers

All 9 C. jejuni strains were resistant to ciprofloxacin and erythromycin. Ciprofloxacin resistance was granted by mutations in the GyrA amino acid sequence at position 86 (T86I (n=8) and T86R (n=1)), and erythromycin resistance was attributed to diverse 23S rDNA mutations: A2074C (n=2), A2074T (n=3), A2074G (n=1) and A2075G (n=3) (Tab. 2). A total of 5 strains (56%) were resistant to
tetracycline, attributed to the presence of tetO, and 2 strains (22%) were resistant to ampicillin conferred by the G63T mutation in the OXA61 promoting region.

Among the 42 multiresistant C. coli strains, all strains were resistant to ciprofloxacin, erythromycin and tetracycline, 41 strains (97.6%) were also resistant to ampicillin, and 6 strains (14.3%) were resistant to gentamicin (Tab. 2). Similar to C. jejuni, mutation T86I in the QRDR region of the GyrA protein was found in all ciprofloxacin-resistant strains, and an additional D90N mutation was found in 2 of them (4.8%). Resistance to tetracycline has been attributed to the presence of tetO (42) for all strains and resistance to ampicillin to a single mutation (G63T) in the promoter region of OXA61 β-lactamase (31) in almost all ampicillin-resistant strains but one (2018/2697). Nucleotide alignment revealed an entirely different promoter region compared to that of other analyzed C. coli genomes. This sequence is described in one ampicillin-resistant Chinese strain (HS11B, KX272768.1) (43) in the NCBI database, but unfortunately no MIC was determined in this study. Further investigations are needed to determine whether any mutation can be associated with resistance. Multiple versions of the APH(2") gene have also been identified and associated with gentamicin resistance in corresponding strains (n=6, all C. coli) (12). These include APH(2")-If, APH(2")-Ih, APH(2")-IIIa, APH(2")-Ic and AAC(6')-Ie-APH(2")-Ia.

Finally, mutations in 23S rDNA were found in 38 erythromycin-resistant strains (90.5%) (44): 36 strains had the A2075G mutation (85.7%), and 2 had the A2074G mutation (4.8%). Two 23S methyltransferases have been identified among the remaining 4 erythromycin-resistant strains from 2019. Protein sequence alignment (Fig. 2) revealed the presence of erm(B) (n=1 strain; 2019/0773), a methyltransferase mainly described in China (45) (46) (47) and located within Type III Multidrug Resistance Genomic Island (MDRGI) (Fig. 3) (48), and a novel 23S methyltransferase named erm(N) for convenience in the present study (n=3 strains; 2019/0051, 2019/0191 and 2019/2001), described
only once in Quebec (17) and found within the CRISPR array of the CRISPR-cas9 operon (Fig. 4). Only C. coli clade 1 of the Type 2 CRISPR-Cas system was found in the present study, composed of cas9-1-2, CRISPR repeats/spacers and ending with the moeA gene (49). PCR screening was performed on erythromycin-resistant strains from our collection to estimate the prevalence of these methylases.

**PCR screening of erm(B) and erm(N) in erythromycin-resistant strains**

PCR screening of erm(B) and erm(N) among 171 erythromycin-resistant Campylobacter strains revealed 5 new methyltransferase-positive C. coli strains. Three strains from 2016 (2016/0940, 2016/2392 and 2016/0429H) were positive for erm(N) two years before the first report in Quebec, and 2 strains from 2017 and 2018 (2017/0180 and 2018/1149) were positive for erm(B). In summary, from 2016 to 2019, 1.4% (n=3) of all erythromycin-resistant strains from our collection were erm(B)-positive, and 2.7% (n=6) were erm(N)-positive. WGS of erm(N)-positive strains showed conserved CRISPR-cas9 regions (Fig. 4): sequences surrounding the CRISPR array and erm(N) sequences were identical. Similar spacers within the CRISPR array were also found among each strain but in different orders. However, WGS of erm(B)-positive strains from 2017 and 2018 revealed diverse genomic regions (Fig. 3). Genomic analyses revealed the presence of a Type III MDRGI in one C. coli strain (2019/0773), highly similar to the ZP-GX-1 Chinese strain (KC876748.1) (48). Two other erm(B)-positive C. coli strains identified by PCR screening (2017/0180 and 2018/1149) did not display any defined MDRGI type. In strain 2017/0180, erm(B) is carried by a plasmid (47.6 kbp contig length) within a standard resistance genomic island structure found in C. coli p1CFSAN032805 (CP045793.1, 55 kbp length), which was identified in Spain in 2019 (8) from humans, animals and sewage strains, or in the C. jejuni pGMI16-002 plasmid (CP028186.1, 66.6 kbp length). Despite their great similarity based on the presence of multiple resistance markers against gentamicin (APH(3’)-IIIa), streptomycin (sat4), aminoglycosides (aadK and ANT(9)) and tetracycline (tetO), both plasmids initially did not
harbor any methyltransferase. Gene transfer of chromosomal \textit{erm(B)} within mobile MDRGI may have occurred and could now disseminate more easily. On the other hand, \textit{C. coli} 2018/1149 \textit{erm(B)} was found within a chromosomal MDRGI among duplicate copies of \textit{tetO} and \textit{ANT(9)}, similar to the 16SHKX65C \textit{erm}-positive strain (CP038868.1) reported in 2016 in China (50).

\textbf{Transformation of the CRISPR-\textit{erm(N)} PCR product transfers erythromycin resistance}

Bacterial transformations with CRISPR-\textit{erm(N)} PCR products into a CRISPR-\textit{cas9}-positive, \textit{erm(N)}-negative, 23S rDNA mutation-free and erythromycin-sensitive \textit{C. coli} isolate were successfully performed. The presence of \textit{erm(N)} in receiver strains was confirmed by PCR screening using primers designed on both sides of the expected integration site (Fig. 5). Moreover, WGS of transformants (2019/0231H/0051 and 2019/0231H/0191) revealed that the insertion occurred at the locus by strict homologous recombination (Fig. 6). \textit{erm(N)} insertion into the CRISPR array was followed in both transformants by a significant increase in erythromycin MICs, from 1.5 µg/mL to 64 µg/mL before and after transformation, respectively. However, bacterial transformations showed here that MIC values were not strictly conserved after the insertion of CRISPR-\textit{cas9-erm(N)} products. Donor isolate 2019/0191 initially displayed high level of resistance (MIC ≥256 µg/mL) whereas transformant 2019/0231H/0191 showed a lower level of 64 µg/mL. Similarly, transformant 2019/0231H/0051 showed a 4-fold difference in erythromycin MIC with its initial donor (64 µg/mL against 16 µg/mL for 2019/0051 donor isolate). Finally, no mutation was detected in the 23S rDNA sequence of the transformants, which led to the conclusion that \textit{erm(N)} expression is solely associated with erythromycin resistance.

In conclusion, with the application of our WGS strategy to multiresistant \textit{C. coli} and \textit{C. jejuni} strains, \textit{erm(B)} and \textit{erm(N)} were identified for the first time in France in \textit{C. coli} strains isolated from human cases. \textit{erm(N)} corresponds to a new methyltransferase never before described in Europe.
DISCUSSION

Resistance to antibiotics is a matter of concern worldwide. Since the discovery of these molecules, bacteria have learned to adapt to their environment and have developed various defense mechanisms. Campylobacter species are of particular concern, and resistance has increased over the past few years. Strong selective pressure has revealed various resistance mechanisms among Campylobacter sp. genomes. Here, we were interested in resistance genes and mutations of multiresistant C. jejuni and C. coli French strains that were selected from clinical cases between 2018 and 2019. We have shown that WGS and bioinformatics tools combined with public and in-house databases are powerful tools for retrieving well-known mutations or genes associated with ampicillin, ciprofloxacin, tetracycline, erythromycin and gentamycin resistance. The bioinformatics approach implemented in the present study also allowed us to identify erythromycin resistance-associated methyltransferases for the first time in France. Among 9 C. coli isolates, there were two distinct methyltransferases, \( \text{erm}(B) \) and \( \text{erm}(N) \), which are shared by various bacterial species. Erm enzymes are able to methylate 23S ribosomal RNA to decrease the binding of macrolides.

\( \text{erm} \)-expressing Campylobacter strains were mainly described in China, with \( \text{erm}(B) \) being the main methyltransferase encountered and commonly found within MDRGIs. \( \text{erm}(B) \) was reported for the first time in 2014 (46) within a C. coli strain from 2008 and multiple times until now in this country or provinces (45) (47). The first written record of an \( \text{erm}(B) \)-positive isolate outside of China was in Kenya in 2013 (52), and since then, various countries have reported their first cases, such as in Mongolia in 2015 (53), the USA in 2018 (54) or Australia in 2020 (55). In Europe, \( \text{erm}(B) \) was reported in C. coli from broilers in Belgium and Spain in 2012 and 2015, respectively (15) (16). It is now accepted that Campylobacter erythromycin-resistant isolates may express \( \text{erm}(B) \). Screening for this gene is performed in some laboratories interested in Campylobacter sp. from animal origins.
Despite its emergence, some countries have failed to identify its presence, such as France, Poland or Korea. Although \(erm(B)\) can be carried by a plasmid or harbored in chromosomal regions, its nucleotide sequences remain highly identical between each isolate, indicating a quick and straightforward dissemination between \textit{Campylobacter} species. To the best of our knowledge, this current paper describes the first \(erm(B)\)-positive \textit{Campylobacter coli} strains in France.

While most studies tend to focus on \(erm(B)\), the second completely different Erm identified in the present study remained undetected until its first and only report in 2018 in a Canadian \textit{C. coli} strain (17). That novel Erm, called here “Erm(N)”, was exclusively located within CRISPR arrays. It is clear that the CRISPR-cas9 immune system is somehow related to the presence of an antibiotic resistance gene within its region. However, the role of CRISPR-cas9 in bacterial adaptation and gene acquisition remains obscure, and no consensus has been reached yet. A recent study suggested that CRISPR-cas9 restrains horizontal gene transfer (HGT) in \textit{Pseudomonas aeruginosa} by targeting phages that may contain exogenous bacterial DNA sequences (59). It has also been shown that CRISPR-cas9 can inhibit conjugation and transformation by interfering with foreign DNA containing adaptive material such as virulence or antibiotic resistance genes (60) (61). In line with this finding, we failed to observe bacterial conjugations between CRISPR-cas9-\(erm(N)\) strains and bacteria with or without CRISPR-cas9 (data not shown). In contrast, we demonstrated in the present study that bacterial transformation is possible with the transfer of \(erm(N)\) PCR products. A very recent study has also shown that the entire CRISPR-cas9 system can be acquired by one bacterium through transduction (62).

Moreover, highly identical \texttt{cas1-2-9, erm(N)} and CRISPR spacer sequences were identified in all positive \textit{C. coli} strains, suggesting that the whole region could have been transferred between bacteria. It is also expected that the entire region can be transferred vertically between cells since the
region is chromosomal. However, we have shown from bacterial transformations that erythromycin MICs can vary although CRISPR-cas9-erm(N) region is conserved, suggesting expression level of erm(N) may be mediated by unrelated CRISPR-cas9 factors. In fact, diverse genomic factors may be involved as insinuated by SNP phylogeny (Figure 1), where high MIC (≥ 256 µg/mL) erm(N) positive strains are located within the same distinct cluster in contrast with lower MIC strains. Regulation of erm(N) could be investigated in further analyses using qPCR on a higher number of positive isolates. The presence of CRISPR-cas9-erm(N) is also rare and involves only 2.7% of our erythromycin-resistant strains. It has only been found in C. coli, where the proportion of CRISPR-cas9-positive strains is relatively low. Consistent with a previous study (49), less than 8% of all C. coli in our genome collection (n=227) harbored this particular immune system and, comparatively, accounted for approximately 90% of C. jejuni strains (n=247) (analysis performed using BLAST and cas sequences; data not shown). We therefore suggest that the presence of the CRISPR-cas9 operon within the C. jejuni genome may have a negative impact on the transfer of erm(N) itself. The insertion of erm(N) in C. coli through recombination may have occurred punctually and, since then, mostly disseminated using CRISPR-cas9 as a vector to transfer to bacteria without such an immune system.

CRISPR-cas9 interference in conjugation and transformation may explain why erm(N) has slowly disseminated since at least 2016 in France. Even though time is not a major factor, Erm methyltransferases have become an issue of considerable concern, especially erm(B) in China (45) (48), and every other country should not underestimate such antibiotic resistance mechanisms. To date, erm(N)-positive strains have only been identified in Quebec and France, but it is more likely this methyltransferase is circulating elsewhere in the world. Therefore, in addition to erm(B) PCR screening, research on erm(N) in new and older strains needs to be undertaken. Source attribution based on host-segregating SNPs also highlights the importance of the chicken reservoir in its diffusion, and specific monitoring in broiler production units may be considered. The emergence of
these new resistance mechanisms has to be followed carefully in the coming years and in any strain collections, either from humans or animals.
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Table 1. Campylobacter jejuni and coli strains and genomes used in the study

Metadata for all strains and genomes analyzed in this study. Assembled sequences are available in both BIGSdb and NCBI databases using their ID\(^1\) and BioSample\(^2\) numbers, respectively. Antimicrobial susceptibility\(^3\) was determined by the disk diffusion method (see Materials and Methods) (AMP, ampicillin; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; GEN, gentamicin): R for resistant and S for susceptible. Campylobacter species were determined by MALDI-TOF. Sex and age of the patients are indicated. Clonal complexes (CC) and sequence types (ST) determined by MLST are shown. Source of contamination estimation\(^4\) was performed using STRUCTURE software combined with 15 source-discriminating genes\(^{(36)}\) and 259 SNPs\(^{(37)}\) for C. jejuni and C. coli, respectively.

Table 2. Antimicrobial resistance profiles and resistance mechanisms identified by NGS

Resistance mechanisms identified by NGS for the 51 Campylobacter strains studied were identified using Ariba v2.14.4 software combined with CARD v3.1.0 and local Campylobacter resistance mechanism databases (see Material and Methods). The corresponding number of isolates\(^1\) for each resistance profile is indicated.

AMP, ampicillin; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; GEN, gentamicin; “-”, no resistance gene, strain susceptible to the corresponding molecule.

Figure 1. Whole Genome SNP phylogenetic tree based on NTICC13 C. coli reference and from every C. jejuni (n=9) and C. coli (n=50) analyzed genomes
Analyses were performed from assembly fasta files on which species identification was assessed from 33 *Campylobacter* species: *C. armoricus, avium, blaseri, canadensis, coli, concisus, corcagiensis, cuniculorum, curvus, fetus, geochelonis, gracilis, helveticus, hepaticus, hominis, hyointestinalis, iguaniorum, insulaenigrae, jejuni, lanienae, lari, mucosalis, novaezeelandiae, ornithocola, peloridis, pinnipediorum, rectus, showae, sputorum, subantarctic*, *upsaliensis, ureolyticus and volucris*. Two distinct clades for both species were obtained based on the analysis of 220,825 SNPs from the alignment on NTICC13 *C. coli* reference genome. Branch lengths display total number of SNP difference in percentage. Highlighted isolates indicate sequence types, and vertical colored bars clonal complexes. Gray STs or CCs are unknown or unique types. Corresponding *erm*-positive strains are indicated on the right side along with associated MIC for erythromycin. Here, *C. coli* isolates show less diverse ST and CC and highly similar genomes, especially at the top of the tree, contrary to *C. jejuni* isolates. Moreover, 3 clusters of *erm*(N)-positive *C. coli* isolates are displayed depending on their MIC values (16, 64 and ≥ 256 µg/mL), suggesting diverse genomic factors may be involved in the mediation of *erm*(N) expression.

**Figure 2.** Phylogenetic tree from amino acid sequence alignment of erythromycin resistance methyltransferases

Alignments were performed using Muscle v3.8.1551 and 5 Erm versions: Erm(B), (D), (F), (N) and a putative Erm from *C. jejuni* 11168. Highlighted isolates correspond to positive *C. coli* for Erm(B) and Erm(N) identified in this study. Strain names are displayed between “[]” and accession numbers between “()” when WGS was not performed. Each clade is distinct from each other, except Erm(D), which is located close to Erm(F). Moreover, Erm(B) sequences and Erm(N) sequences are highly similar between each strain and country.
Figure 3. Multidrug resistance genomic islands (MDRGIs) of *erm*(B)-positive strains

Each *erm*(B)-positive isolate in our collection (n=3) was aligned against various MDRGI types described in previous publications. Gene similarities are indicated here as percentages and using “*” and “**” when genes are distant. MDRGI found in *C. coli* isolate 2019/0773 (SAMN18478599) showed high similarity to Type III MDRGI described in China (48), whereas in *C. coli* isolates 2017/0180 (SAMN18478618) and 2018/1149 (SAMN18478619), their respective MDRGIs did not correspond to any defined type. In strain 2017/0180, *erm*(B) is carried by a plasmid (47.6 kbp contig length) within a standard resistance genomic island structure found in *C. coli* p1CFSAN032805 (CP045793.1, 55 kbp length) identified in Spain in 2019 (8) but without any methyltransferase. *C. coli* 2018/1149 *erm*(B) is expressed within a chromosomal MDRGI among duplicate copies of *tetO* and *ANT(9)*, similar to the 16SHKX65C *erm*(B)-positive strain (CP038868.1) reported in 2016 in China (50).

Figure 4. Alignment of CRISPR-cas9 operons in *erm*(N)-positive strains

CRISPR-cas9 regions of each *erm*(N)-positive isolate were extracted from WGS (displayed order: 2019/0191: SAMN18478591; 2019/2001: SAMN18478607; 2019/0051: SAMN18478589; 2016/0940: SAMN18478616; 2016/2392: SAMN18478617; 2016/0429H: SAMN18478615) between the cas9 and moeA genes. CRISPR arrays are indicated in orange and yellow boxes for *C. coli* palindromic repeat sequences (ATTTTACCATAAAGAAATTAAAAAGGGACTAAAA and ATTTTACCATAAGAAATTAAAAAGGGACTAACCC, respectively) and boxed numbers for viral/plasmid spacers. Similar spacers were found in every isolate but in different configurations. Moreover, sequences for cas9, cas1, cas2, *erm*(N) and *moeE* were 100% identical between all isolates.
Figure 5. *erm*(N) and CRISPR-cas9 PCR from WT and transformant isolates


2% agarose gel, 100 bp and 1 kbp ladders on the left and right sides, respectively.

Figure 6. Region of CRISPR-*erm*(N) PCR product insertion into erythromycin-sensitive and CRISPR-cas9-positive *C. coli*

Aligned CRISPR-*erm*(N) regions were extracted from WGS data of *erm*(N)-positive *C. coli* 2019/0191 (SAMN18478589), WT sensitive strain 2019/0231H (SAMN18478624) and a transformed isolate (SAMN18478621), and are displayed in lanes #1 for the WT sequence, #2 for the PCR product used for transformation and #3 for the transformant sequence. Highlighted sequences indicate *cas2* in blue, CRISPR arrays in green, *erm*(N) in red, *moeA* in yellow and forward and reverse primers in purple. For display purposes, the left CRISPR array was cut between 493 and 1000 bp, although fully identical between the transformed isolate and PCR product.
**Enterococcus faecium** [e389] JAPAN 2002
- **Campylobacter coli** [ZTA09/02204] SPAIN 2015
- **Campylobacter jejuni** [C179b] CHINA 2012
- **Campylobacter coli** [ZC113] CHINA 2008
- **Lactobacillus plantarum** [pLFE1]
  - **Enterococcus faecium** [e226] JAPAN 1998
- **Campylobacter coli** [2019/0773] FRANCE 2019
- **Streptococcus suis** [BM407]
- **Campylobacter coli** (acc=WP_060794049.1) Erm(B) REF 2019
  - **Bacteroides vulgatus** (acc=A0A662ZT84) Erm(D)
  - **Bacteroides fragilis** (uni=P10337) Erm(F)
  - **Uncultured bacterium** [ERI9] (acc=MG585955.1)
- **Campylobacter coli** [48777] QUEBEC 2018
  - **Campylobacter coli** [2019/001] FRANCE 2019
  - **Campylobacter coli** [2019/0191] FRANCE 2019
  - **Campylobacter coli** [2019/0051] FRANCE 2019
- **Campylobacter jejuni** [11168] new methyltransferase
<table>
<thead>
<tr>
<th>Isolate name</th>
<th>BSIDb</th>
<th>NCBI</th>
<th>Species</th>
<th>Patient sex</th>
<th>Patient age</th>
<th>Antimicrobial susceptibility</th>
<th>ST</th>
<th>CC</th>
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Table 1. Campylobacter jejuni and coli strains and genomes used in the study
### Table 2. Antimicrobial resistance profiles and resistance mechanisms identified by NGS

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<th>Species</th>
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<th>TET</th>
<th>GEN</th>
<th>Resistance markers</th>
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**Note:** The table includes antimicrobial resistance profiles and resistance mechanisms identified by NGS. The resistance mechanisms include blaOXA-61, tetO, and combinations of erm and APH(2'')-Ic and AAC(6')-Ie-APH(2'')-Ia. The specific mutations in the 23S rRNA gene and the gyrA gene are also highlighted in the table.