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) (www.cnrch.fr). 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% N_2, 5–10% CO_2, and 5–10% H_2. 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'-AAAGCCATGCGTCTGACATC-3' (F) and 5'-CTTACCCGCCATACCACAGA-3' (R) with an expected PCR product size of 196 bp and primers for *erm(N)* 5'-TAGCGGTTACAGACGAGTCC-3' (F) and 5'-GATTCTGGCATTGGGTACGG-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 *erm(B)* within mobile MDRGI may have occurred and could now disseminate more easily. On the other hand, *C. coli* 2018/1149 *erm(B)* was found within a chromosomal MDRGI among duplicate copies of *tetO* and *ANT(9)*, similar to the 16SHKX65C *erm*-positive strain (CP038868.1) reported in 2016 in China (50).

Transformation of the CRISPR-*erm(N)* PCR product transfers erythromycin resistance

Bacterial transformations with CRISPR-*erm(N)* PCR products into a CRISPR-*cas9*-positive, *erm(N)*-negative, 23S rDNA mutation-free and erythromycin-sensitive *C. coli* isolate were successfully performed. The presence of *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). *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-*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 *erm(N)* expression is solely associated with erythromycin resistance.

In conclusion, with the application of our WGS strategy to multiresistant *C. coli* and *C. jejuni* strains, *erm(B)* and *erm(N)* were identified for the first time in France in *C. coli* strains isolated from human cases. *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, *erm(B)* and *erm(N),* which are shared by various bacterial species. Erm enzymes are able to methylate 23S ribosomal RNA to decrease the binding of macrolides (51).

erm-expressing *Campylobacter* strains were mainly described in China, with *erm(B)* being the main methyltransferase encountered and commonly found within MDRGIs. *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 *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, *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 *erm(B).* Screening for this gene is performed in some laboratories interested in *Campylobacter sp.* from animal origins (2)

(56). Despite its emergence, some countries have failed to identify its presence, such as France (56), Poland (57) or Korea (58). 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 *Campylobacter* species. To the best of our knowledge, this current paper describes the first *erm(B)*-positive *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 *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 *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 *cas1-2-9*, *erm(N)* and CRISPR spacer sequences were identified in all positive *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 varied 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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374 **Legends**

375

376 **Table 1. *Campylobacter jejuni* and *coli* strains and genomes used in the study**

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

386

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

388 Resistance mechanisms identified by NGS for the 51 *Campylobacter* strains studied were identified
389 using Ariba v2.14.4 software combined with CARD v3.1.0 and local *Campylobacter* resistance
390 mechanism databases (see Material and Methods). The corresponding number of isolates[1] for each
391 resistance profile is indicated.
392 AMP, ampicillin; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; GEN, gentamycin; "-",
393 no resistance gene, strain susceptible to the corresponding molecule.

394

395 **Figure 1. Whole Genome SNP phylogenetic tree based on NTICC13 *C. coli* reference and from**
396 **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, subantarcticus, 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 bewteen "()" 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 (ATTTTACCATAAAGAAATTTAAAAAGGGACTAAAA and ATTTTACCATAAAGAAAATTAAAAAGGGACTAACCC, 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 *moaE* were 100% identical between all isolates.

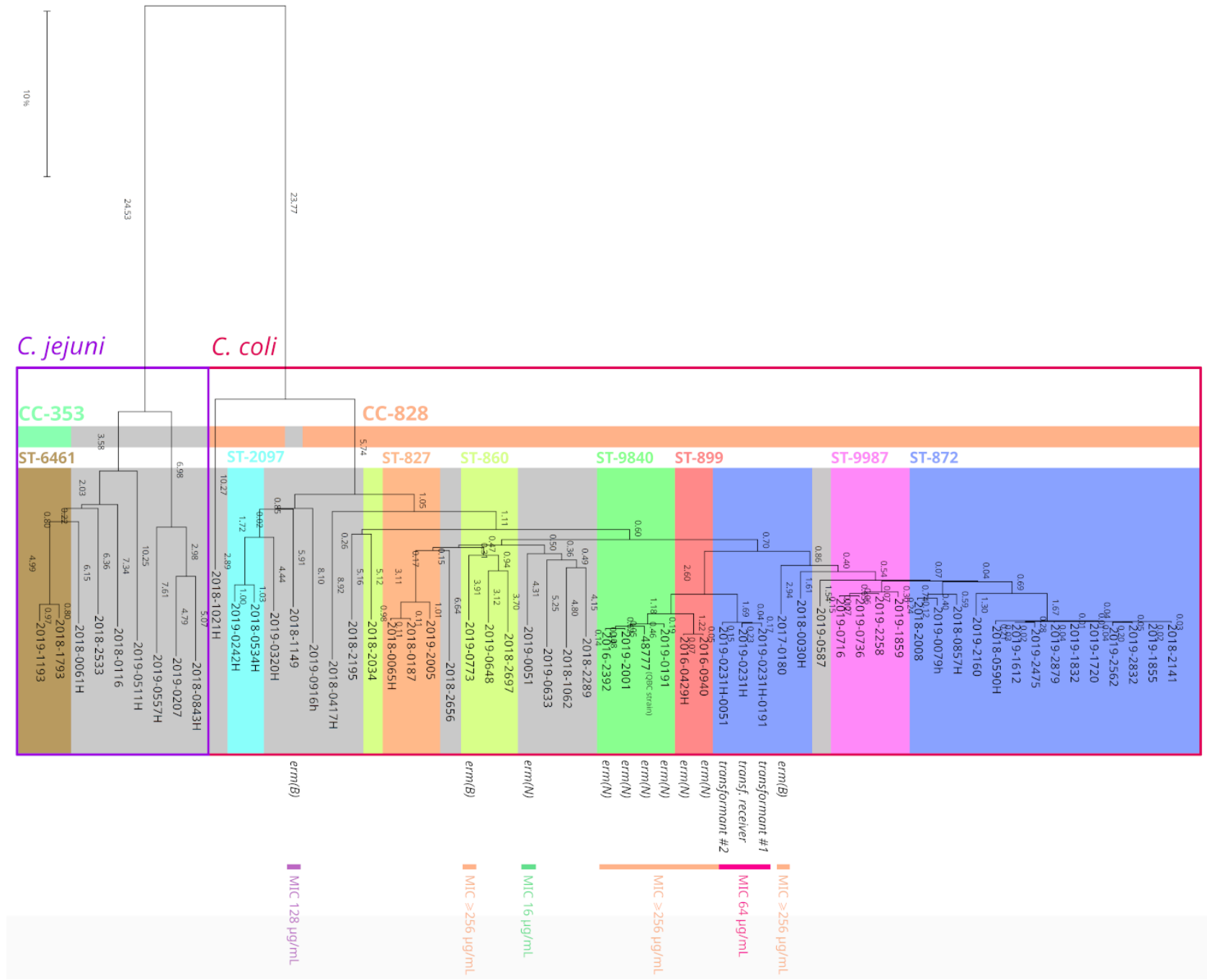
Figure 5. *erm(N)* and CRISPR-*cas9* PCR from WT and transformant isolates

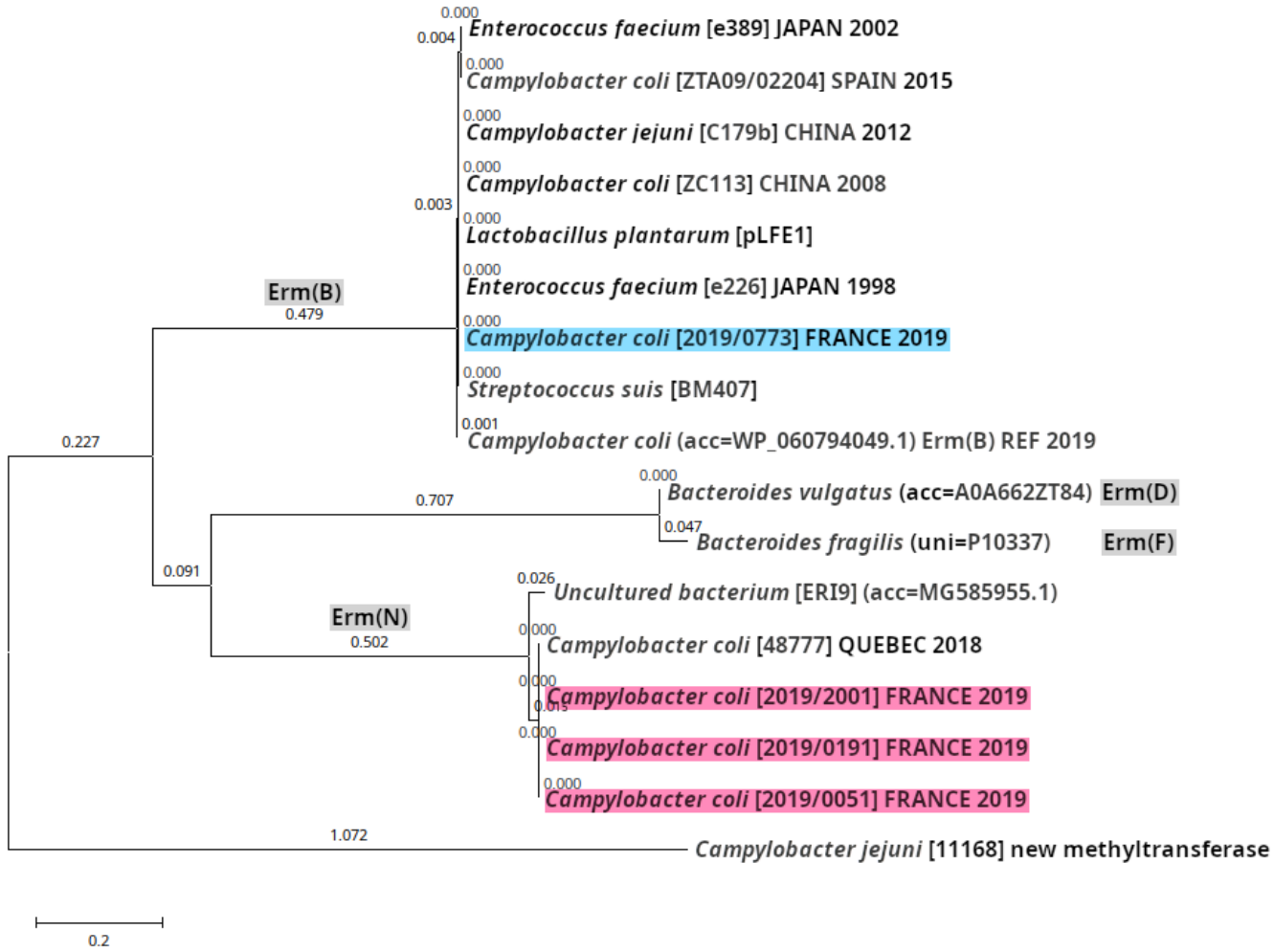
(1) *erm(N)*-PCR negative, strain 2019/0231H (SAMN18478624) control, **(2)** *erm(N)*-PCR positive control, strain 2019/0191 (SAMN18478591), **(3)** *erm(N)*-PCR positive control, strain 2019/0051 (SAMN18478589), **(4)** *erm(N)*-PCR, transformant 2019/0231H/0191 (SAMN18478621), **(5)** *erm(N)*-PCR, transformant 2019/0231H/0051 (SAMN18478620), **(6)** CRISPR-*cas9*-PCR negative control (blank), **(7)** CRISPR-*cas9*-*erm(N)*-PCR control, strain 2019/0191, **(8)** CRISPR-*cas9 erm(N)*-PCR control, strain 2019/0051, **(9)** CRISPR-*cas9*-PCR, strain 2019/0231H, **(10)** CRISPR-*cas9* PCR strain 2019/0231H/0191, **(11)** CRISPR-*cas9* PCR strain 2019/0231H/0051.

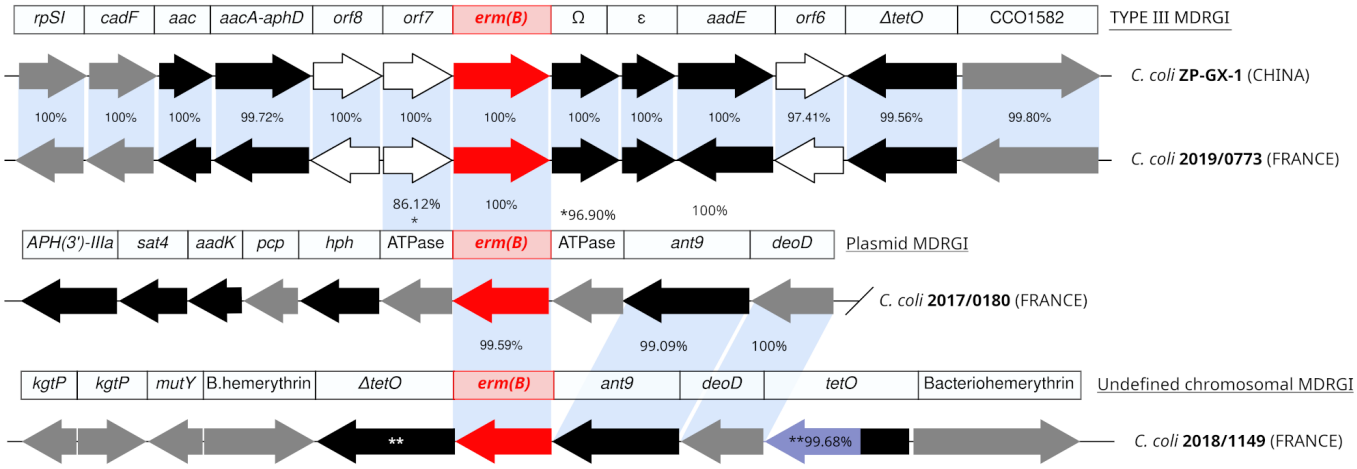
2% agarose gel. 100 bp and 1 kbp ladders on the left and right sides, respectively.

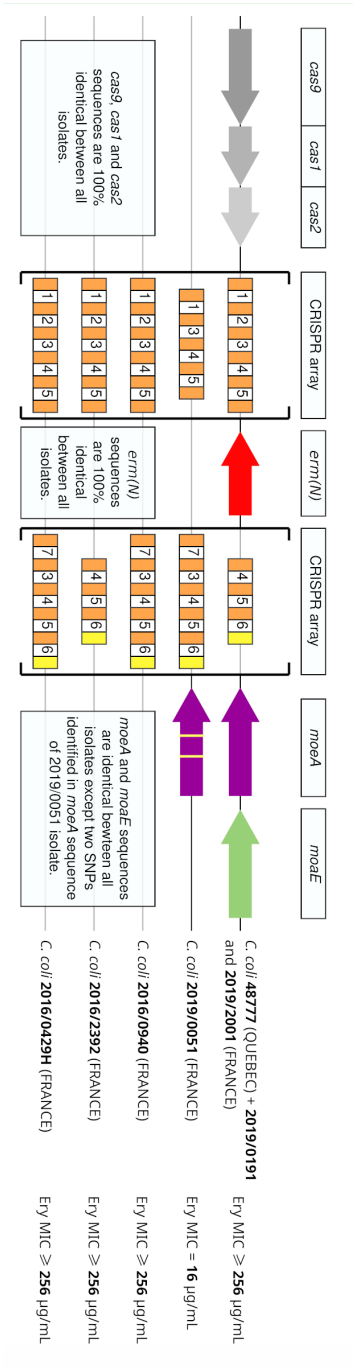
Figure 6. Region of CRISPR-*erm(N)* PCR product insertion into erythromycin-sensitive and CRISPR-*cas9*-positive *C. coli*

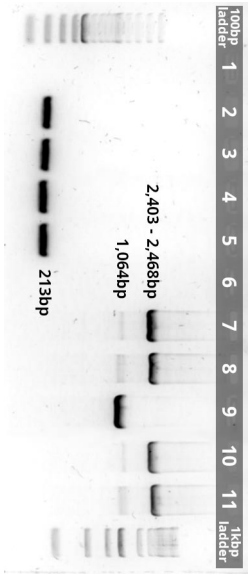
Aligned CRISPR-*erm(N)* regions were extracted from WGS data of *erm(N)*-positive *C. coli* 2019/0191 (SAMN18478589), WT sensitive strain 2019/0231H (SAMN18478624) and a transformed isolate (SAMN18478621), and are displayed in lanes #1 for the WT sequence, #2 for the PCR product used for transformation 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.

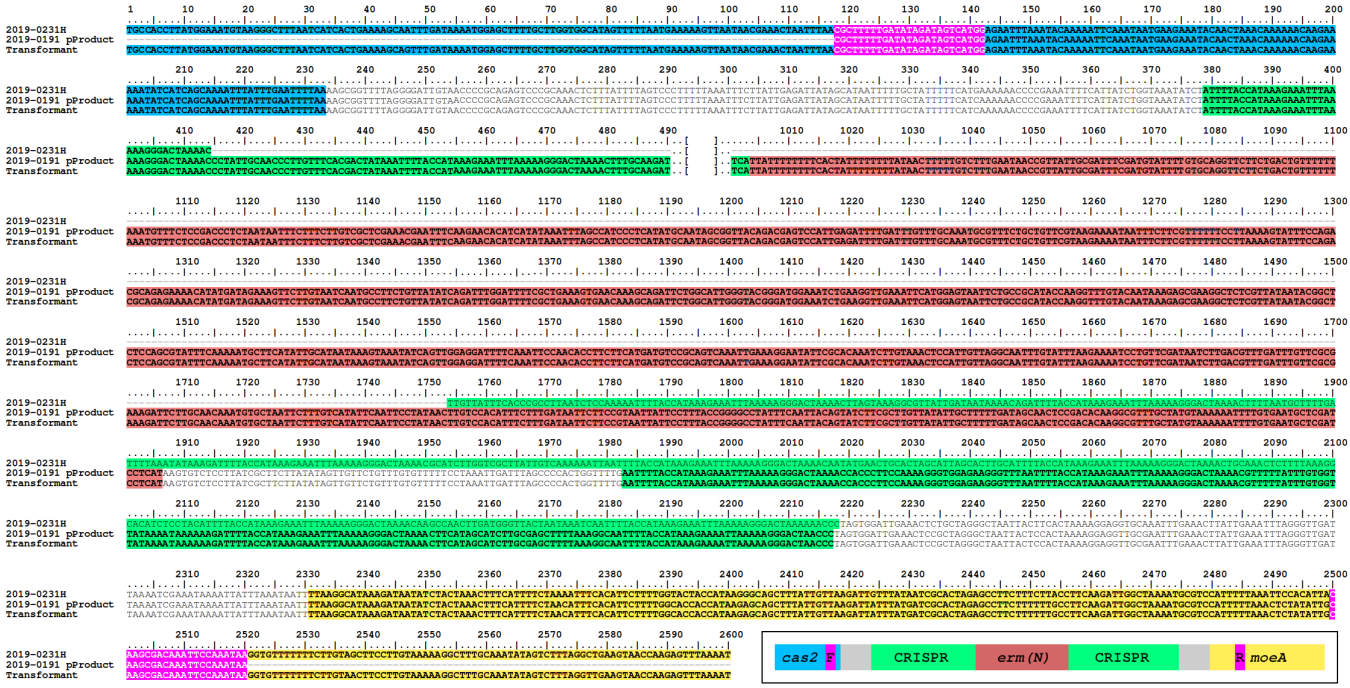












Isolate name	BIGSdb[1]	NCBI[2]	Species	Patient sex	Patient age	Antimicrobial susceptibility[3]					ST	CC	Source[4]
						AMP	CIP	ERY	TET	GEB			
2018/0061H	12577	SAMN18478564	*C. jejuni*	F	67	S	**R**	**R**	S	S	2066	ST-52	chicken
2018/0116	12525	SAMN18478565	*C. jejuni*	M	68	S	**R**	**R**	**R**	S	2274	-	chicken
2018/0843H	12530	SAMN18478566	*C. jejuni*	M	43	S	**R**	**R**	**R**	S	-	-	environment
2018/1793	12535	SAMN18478567	*C. jejuni*	F	74	**R**	**R**	**R**	**R**	S	6461	ST-353	chicken
2018/2533	12541	SAMN18478568	*C. jejuni*	F	51	I	**R**	**R**	**R**	S	2328	-	chicken
2019/0207	12548	SAMN18478569	*C. jejuni*	M	59	S	**R**	**R**	**R**	S	7010	-	environment
2019/0511H	12551	SAMN18478570	*C. jejuni*	M	59	S	**R**	**R**	**R**	S	6532	ST-42	cattle
2019/0557H	12693	SAMN18478571	*C. jejuni*	M	60	S	**R**	**R**	S	S	6	-	environment
2019/1193	12562	SAMN18478572	*C. jejuni*	M	46	**R**	**R**	**R**	**R**	S	6461	ST-353	chicken
2018/0030H	12523	SAMN18478573	*C. coli*	F	81	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2018/0065H	12524	SAMN18478574	*C. coli*	M	15	**R**	**R**	**R**	**R**	S	827	ST-828	chicken
2018/0187	12526	SAMN18478575	*C. coli*	M	15	**R**	**R**	**R**	**R**	S	827	ST-828	chicken
2018/0417H	12527	SAMN18478576	*C. coli*	M	47	**R**	**R**	**R**	**R**	S	-	-	chicken
2018/0534H	12528	SAMN18478577	*C. coli*	M	2	**R**	**R**	**R**	**R**	S	2097	ST-828	pig
2018/0590H	12529	SAMN18478578	*C. coli*	F	82	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2018/0857H	12531	SAMN18478579	*C. coli*	F	66	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2018/1021H	12533	SAMN18478580	*C. coli*	M	59	**R**	**R**	**R**	**R**	S	-	-	pig
2018/1062	12534	SAMN18478581	*C. coli*	M	10	**R**	**R**	**R**	**R**	S	5380	ST-828	chicken
2018/2008	12536	SAMN18478582	*C. coli*	M	2	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2018/2034	12537	SAMN18478583	*C. coli*	M	18	**R**	**R**	**R**	**R**	S	860	ST-828	chicken
2018/2141	12538	SAMN18478584	*C. coli*	M	45	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2018/2195	12539	SAMN18478585	*C. coli*	M	47	**R**	**R**	**R**	**R**	S	1600	ST-828	chicken
2018/2289	12540	SAMN18478586	*C. coli*	F	24	**R**	**R**	**R**	**R**	S	-	-	chicken
2018/2656	12543	SAMN18478587	*C. coli*	F	28	**R**	**R**	**R**	**R**	S	1108	ST-828	pig
2018/2697	12544	SAMN18478588	*C. coli*	F	61	**R**	**R**	**R**	**R**	**R**	860	ST-828	chicken
2019/0051	12545	SAMN18478589	*C. coli*	F	6	**R**	**R**	**R**	**R**	S	-	-	chicken
2019/0079h	12546	SAMN18478590	*C. coli*	M	16	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/0191	12547	SAMN18478591	*C. coli*	M	4	**R**	**R**	**R**	**R**	**R**	9840	ST-828	chicken
2019/0242H	12549	SAMN18478592	*C. coli*	F	16	**R**	**R**	**R**	**R**	**R**	2097	ST-828	pig
2019/0320H	12550	SAMN18478593	*C. coli*	M	25	**R**	**R**	**R**	**R**	S	828	ST-828	pig
2019/0587	12553	SAMN18478594	*C. coli*	M	37	**R**	**R**	**R**	**R**	S	6689	ST-828	chicken
2019/0633	12554	SAMN18478595	*C. coli*	M	57	**R**	**R**	**R**	**R**	S	10048	ST-828	chicken
2019/0648	12555	SAMN18478596	*C. coli*	M	18	**R**	**R**	**R**	**R**	**R**	860	ST-828	chicken
2019/0716	12556	SAMN18478597	*C. coli*	F	21	**R**	**R**	**R**	**R**	S	9987	ST-828	chicken
2019/0736	12557	SAMN18478598	*C. coli*	M	5	**R**	**R**	**R**	**R**	S	9987	ST-828	chicken
2019/0773	12558	SAMN18478599	*C. coli*	M	57	**R**	**R**	**R**	**R**	**R**	860	ST-828	chicken
2019/0916h	12559	SAMN18478600	*C. coli*	F	72	**R**	**R**	**R**	**R**	S	-	-	pig
2019/0973H	12561	SAMN18478601	*C. coli*	M	14	**R**	**R**	**R**	**R**	S	9987	ST-828	chicken
2019/1612	12564	SAMN18478602	*C. coli*	F	14	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/1720	12565	SAMN18478603	*C. coli*	F	8	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/1832	12566	SAMN18478604	*C. coli*	F	41	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/1855	12567	SAMN18478605	*C. coli*	F	56	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/1859	12568	SAMN18478606	*C. coli*	M	7	**R**	**R**	**R**	**R**	S	9987	ST-828	chicken
2019/2001	12569	SAMN18478607	*C. coli*	F	14	**R**	**R**	**R**	**R**	**R**	9840	ST-828	pig
2019/2005	12570	SAMN18478608	*C. coli*	F	87	**R**	**R**	**R**	**R**	S	827	ST-828	chicken
2019/2160	12571	SAMN18478609	*C. coli*	M	18	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/2258	12572	SAMN18478610	*C. coli*	M	59	**R**	**R**	**R**	**R**	S	9987	ST-828	chicken
2019/2475	12573	SAMN18478611	*C. coli*	M	6	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/2562	12574	SAMN18478612	*C. coli*	F	37	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/2832	12575	SAMN18478613	*C. coli*	F	38	**R**	**R**	**R**	**R**	S	872	ST-828	chicken
2019/2879	12576	SAMN18478614	*C. coli*	M	5	**R**	**R**	**R**	**R**	S	872	ST-828	chicken

Table 1. *Campylobacter jejuni* and *coli* strains and genomes used in the study

Species	n of isolates[1]	Resistance markers				
		AMP	CIP	ERY	TET	GEN
C. coli	30	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	23S mutation A2075G	*tetO*	-
C. coli	2	blaOXA-61 promoter mutation G63T	GyrA mutations T86I and D90N	23S mutation A2075G	*tetO*	-
C. coli	2	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	23S mutation A**2074**G	*tetO*	-
C. coli	1	*Sensitive*	GyrA mutation T86I	23S mutation A2075G	*tetO*	-
C. coli	1	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	novel *erm* ***erm(N)***	*tetO*	-
C. coli	1	*Undescribed* blaOXA-61 promoter mutations	GyrA mutation T86I	23S mutation A2075G	*tetO*	*APH(2")-If* , *APH(2")-Ih* and *AAC(6')-Ie-APH(2")-Ia*
C. coli	2	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	novel *erm* ***erm(N)***	*tetO*	*APH(2")-IIIa* and *APH(2")-Ic*
C. coli	1	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	23S mutation A2075G	*tetO*	*APH(2")-IIIa* and *APH(2")-Ic*
C. coli	1	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	23S mutation A2075G	*tetO*	*AAC(6')-Ie-APH(2")-Ia*
C. coli	1	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	*erm(B)*	*tetO*	*AAC(6')-Ie-APH(2")-Ia*
C. jejuni	1	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	23S mutation A2074C	*tetO*	-
C. jejuni	1	blaOXA-61 promoter mutation G63T	GyrA mutation T86I	23S mutation A2075G	*tetO*	-
C. jejuni	2	-	GyrA mutation T86I	23S mutation A2074T	*tetO*	-
C. jejuni	2	-	GyrA mutation T86I	23S mutation A2075G	*tetO*	-
C. jejuni	1	-	GyrA mutation T86I	23S mutation A2074C	*tetO*	-
C. jejuni	1	-	GyrA mutation T86R	23S mutation A2074T	-	-
C. jejuni	1	-	GyrA mutation T86I	23S mutation A2074G	-	-

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