**Novel plasmid conferring kanamycin and tetracycline resistance in the turkey-derived
Campylobacter jejuni strain 11601MD**

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ABSTRACT

In *Campylobacter* spp., resistance to the antimicrobials kanamycin and tetracycline is frequently associated with plasmid-borne genes. However, relatively few plasmids of *Campylobacter jejuni* have been fully characterized to date. A novel plasmid (p11601MD; 44,095 nt) harboring *tet*(O) was identified in *C. jejuni* strain 11601MD, which was isolated from the jejunum of a turkey produced conventionally in North Carolina. Analysis of the p11601MD sequence revealed the presence of a high-GC content cassette with four genes that included *tet*(O) and a putative aminoglycoside transferase gene (*aphA-3*) highly similar to kanamycin resistance determinants. Several genes putatively involved in conjugative transfer were also identified on the plasmid. These findings will contribute to a better understanding of the distribution of potentially self-mobilizing plasmids harboring antibiotic resistance determinants in *Campylobacter* spp. from turkeys and other sources.

Keywords: *Campylobacter*; *C. jejuni*; plasmid; antibiotic resistance; *tet*(O); tetracycline resistance; kanamycin resistance; *aphA-3*

1. Introduction

Campylobacter spp. are leading human foodborne pathogens that frequently colonize poultry and other food animals. *C. jejuni* is responsible for the majority (>85%) of human campylobacteriosis cases, with the remainder primarily associated with *C. coli* (Gillespie *et al.*, 2002 and Scallan *et al.*, 2011). *C. jejuni* and *C. coli* are well known for their ability to acquire resistance to various antimicrobials, with resistance to tetracycline being especially common (Taylor, 1986 and Luangtongkum *et al.*, 2009). The concern exists that antimicrobial-resistant

Campylobacter spp. can be transmitted from colonized food animals to humans through the food chain (Luangtongkum *et al.*, 2009, McCrackin *et al.*, 2015 and Sahin *et al.*, 2015).

In *Campylobacter* spp. resistance to tetracycline is typically associated with plasmid-borne *tet*(O) genes (Taylor *et al.*, 1983, Tenover *et al.*, 1985, Taylor, 1986, Dasti *et al.*, 2007 and Alfredson and Korolik, 2007), but chromosomal *tet*(O) has also been described in *C. jejuni* and *C. coli* (Pratt and Korolik, 2005 and Crespo *et al.*, 2012). *Campylobacter* spp. plasmids harboring *tet*(O) often harbor kanamycin resistance determinants also (Taylor *et al.*, 1983, Kotarski *et al.*, 1986 and Gibreel *et al.*, 2004) and *aphA-3*, encoding a 3'aminoglycoside phosphorylase type III, which has been shown to mediate kanamycin resistance (Gibreel *et al.*, 2004). However, in spite of the important role of plasmids in antimicrobial resistance of *Campylobacter* spp., relatively few plasmids of *C. jejuni* have been fully characterized to date.

Characterization of antimicrobial susceptibility profiles of *C. jejuni* from conventionally grown turkeys revealed that resistance to tetracycline was the most frequently encountered resistance attribute, being encountered among >80% of the isolates, followed by resistance to kanamycin (42.5-76%) (Luangtongkum *et al.*, 2006 and Gu *et al.*, 2009). Strains with kanamycin resistance were typically also resistant to tetracycline, but not *vice versa* (Gu *et al.*, 2009), suggesting acquisition of kanamycin resistance determinants by *tet*(O)-harboring plasmids. In the current study, we sequenced and analyzed a plasmid from *C. jejuni* 11601MD, a strain isolated from the Meckel's Diverticulum (jejunum) of a turkey conventionally raised in eastern North Carolina and exhibiting resistance to multiple antibiotics, including tetracycline, kanamycin, ciprofloxacin and nalidixic acid.

2. Methods

2.1 Bacterial strains and DNA extraction

Turkey-derived *C. jejuni* strains (Table 1) were grown in Mueller-Hinton broth or on Mueller-Hinton agar. Plates were incubated at 42°C for 48 hours under microaerobic conditions using Campy GasPaks (Becton Dickinson and Co., San Jose, CA). Genomic DNA was extracted using DNeasy Blood & Tissue Kits (QIAGEN, Valencia, CA).

Table 1. *Campylobacter jejuni* isolates tested for the presence of p11601MD genes.

Strain	Species	Origin	ST ¹	Antibiotic susceptibility profile ²
11601MD (S1)	<i>C. jejuni</i>	Turkey	2934	TKQ
6532 (S2)	<i>C. jejuni</i>	Turkey	1839	TSQ
7495 (S3)	<i>C. jejuni</i>	Turkey	1796	TK
SC776 (S4)	<i>C. jejuni</i>	Turkey	2934	TK
SC1305 (S5)	<i>C. jejuni</i>	Turkey	467	TK
SC1395 (S6)	<i>C. jejuni</i>	Turkey	1101	TK
SC1925 (S7)	<i>C. jejuni</i>	Turkey	48	TK
10882-C (S8)	<i>C. jejuni</i>	Turkey	1839	TSKQ
2MT8-3 (S9)	<i>C. jejuni</i>	Turkey	ND	TSKQ
WT6-1 (S10)	<i>C. jejuni</i>	Turkey	ND	TSKQ
MT02 (S11)	<i>C. jejuni</i>	Turkey	1839	TSKQ
MT2-4 (S12)	<i>C. jejuni</i>	Turkey	1839	TSKQ
4MT8-8 (S13)	<i>C. jejuni</i>	Turkey	1839	TSKQ
MT14-4 (S14)	<i>C. jejuni</i>	Turkey	2935	TSQ
NCTC 11168 (S15)	<i>C. jejuni</i>	Human	43	N/A

¹ Sequence types (STs) were determined by multi-locus sequence typing. ND, not determined.

² Antimicrobial susceptibility profiles, specifically resistance to tetracycline (T), kanamycin (K), (fluoro)quinolones, i.e. nalidixic acid and ciprofloxacin (Q), and streptomycin (S); e.g., profile TK indicates resistance to tetracycline and kanamycin, but not to the other tested antibiotics (streptomycin, erythromycin, nalidixic acid and ciprofloxacin). Antimicrobial susceptibility profiles were determined as described (Gu *et al.*, 2009). S1-S15 represent strain codes. *C. jejuni* NCTC 11168 (S15) was included as negative control. N/A, not applicable (no known resistance).

2. 2. Sequence analysis

Total genomic DNA of *C. jejuni* strain 11601MD was sequenced at the Genome Core Facility of Duke University, and the annotation of the genome sequence was performed using GAMOLA (Altermann and Klaenhammer, 2003). The presence and comparative genomic location of sequences homologous to the tetracycline resistance gene *tet(O)* in different *Campylobacter* genomes was checked using compACTor (Altermann, 2012) and the ARTEMIS Comparison Tool (Carver *et al.*, 2005). ARTEMIS (Rutherford *et al.*, 2000) was used to visualize the annotated genomes. A plasmid (p11601MD) harbouring genes for resistance to tetracycline and kanamycin, i.e. *tet(O)* and *aphA-3*, respectively, was identified as a single contig. Primers cpp20-5' AAG TAG CCA ATA AAG ATG TTG GTG T-3' and cpp21-5' TGG AGT AAT TTT TCC AAC ACA CTT- 3' were designed to amplify the region between the first and last ORFs in this contig in order to circularize the plasmid sequence.

2. 3. PCR-based detection of plasmid sequences among other turkey-derived *C. jejuni* strains

Turkey-derived *C. jejuni* strains resistant to tetracycline and other antibiotics (Table 1) were tested by PCR using primers derived from p11601MD (Table 2), to determine the presence of nine sequences identified on p11601MD, including: *tet(O)*, *aphA-3*, the intergenic region between *tet(O)* and *aphA-3*, and a panel of other ORFs selected so as to represent different locations on p11601MD. Positive and negative controls (*C. jejuni* strains 11601MD and NCTC 11168, respectively) were included in each PCR. Amplifications were performed using a Biometra T1 thermocycler (Biometra GmbH, Goettingen, Germany) with the following conditions: 0.5 min denaturation at 95°C, 1.0 min annealing at 55°C, and 2.0 min extension at 72°C, with 30 cycles of amplification. For the detection of intergenic region between *aphA-3* and *tet(O)* the extension step was increased to 2.5 min, due to the increased size of the expected PCR product (805 bp).

Table 2. Primers used for PCR detection of selected p11601MD sequences and expected amplicon size

PCR target ¹	Forward Primer (5'-3')	Reverse Primer (5'-3')	Expected amplicon size (nt)
DNA methylase (<i>cpl14</i>) (T1)	GGC GGT CGA AGT TTA TTT GA	CCA TGT CTG TTA GCC CCT GT	335
3' aminoglycoside phosphotransferase type III (<i>aphA-3</i> ; kanamycin resistance) (T2)	GAA AGC TGC CTG TTC CAA AG	ATG TTG CTG TCT CCC AGG TC	382
Tetracycline resistance protein (<i>tet(O)</i>) (T3)	CAA AGG GGA ATC ACT ATC C	AAC CTG CCC GCA TAG TTC	609
DNA topoisomerase III (T4)	GTG GTG GCA TAG GAA CAC CT	GCG GAC ATT GAA TTT TTG GT	333
Type IV secretion system protein, DNA transfer ATPase (VirB11) (T5)	GGT GAA ACA GGA AGC GGT AA	ACG CAT TGA CCT TGT GGA TT	377
Type IV secretion system protein (VirB8) (T6)	AAC CAT GCT TGA TGA AAA AGA AA	TGC TTG AGT TGT TCC TTT GG	331
Type IV secretory pathway (VirB5) (T7)	GGA AAT CTC ATC CCC ATG AA	GCA TCT TTA GCC CAT TCT GC	325
Single-stranded DNA-binding protein (T8)	GGC ACT ACA AAT AGC GGA AGA	GGT GGG ACT TGT GGA TTT TG	314
Type IV secretion/conjugal transfer ATPase (T9)	CGC CCA CGC ATT AAT CTT AT	ATC TAC CGC CAG CAC AAA GT	394
Intergenic region between <i>aphA-3</i> and <i>tet(O)</i> (T10)	AAA GGA TAC CAG CCA GCT ATT	TCA ATT CCG GTG ATA TTC TCA TT	805

¹ Annotations are as indicated in Table 3. T1-T10 are codes for the corresponding PCR-targeted regions.

2.4. Functional genome distribution analysis

A functional genome distribution (FGD) analysis, based on plasmid ORFeomes, was performed with p11601MD and 39 other published *Campylobacter* plasmids using compACTor, MEGA6 (Tamura *et al.*, 2013) and ACT. Publicly available plasmid sequences (n=39) were downloaded in GenBank or FASTA format from the NCBI database. All plasmid sequences were then manually oriented to begin with the same ORF and orientation as the reference

plasmid p11601MD, and subsequently automatically annotated using GAMOLA. Predicted ORFeomes of the 40 plasmids were subjected to FGD analysis (Altermann, 2012) and the resulting distance matrix was imported into MEGA6. The functional distribution was inferred using the UPGMA method (Sneath and Sokal, 1962). The resulting tree was drawn to scale, with branch lengths in the same units as those of the functional distances used to infer the distribution tree.

3. Results and discussion

3.1. Organization of p11601MD and PCR-based detection of homologous sequences among other *C. jejuni* strains

As discussed above, the p11601MD plasmid was detected as a single contig of the whole genome sequence of *C. jejuni* strain 11601MD, and circularized by amplification and sequencing of the region between the first and last ORFs in this contig. The plasmid has a length of 44,095 nt, with a GC content of 29%. The 51 ORFs identified in p11601MD, their size, GC content, and putative annotation are listed in [Table 3](#).

Table 3. ORFs identified in p11601MD

ORF	Size (nt)	GC% ¹	Putative product
<i>p11601MD_01</i>	552	20	cyp18
<i>p11601MD_02</i>	1389	29	Relaxase (cyp17)
<i>p11601MD_03</i>	705	24	Acetyltransferase
<i>p11601MD_04</i>	5796	30	DNA methylase (cyp14)
<i>p11601MD_05</i>	267	27	Hypothetical protein (cyp13)
<i>p11601MD_06</i>	561	25	Hypothetical protein (cyp12)
<i>p11601MD_07</i>	267	23	Hypothetical protein
<i>p11601MD_08</i>	558	25	Hypothetical protein (cyp10)
<i>p11601MD_09</i>	513	25	Hypothetical protein
<i>p11601MD_10</i>	399	22	Hypothetical protein (cyp8)
<i>p11601MD_11</i>	381	22	Hypothetical protein
<i>p11601MD_12</i>	525	48 ¹	Hypothetical protein cco55
<i>p11601MD_13</i>	795	45 ¹	AphA-3 (kanamycin resistance)
<i>p11601MD_14</i>	372	46 ¹	Hypothetical protein

<i>p11601MD_15</i>	1920	40 ¹	Tet(O) (Tetracycline resistance)
<i>p11601MD_16</i>	180	24	Hypothetical protein (cpp51)
<i>p11601MD_17</i>	1056	20	Hypothetical protein (cpp50)
<i>p11601MD_18</i>	2193	29	DNA topoisomerase III (cpp49)
<i>p11601MD_19</i>	621	29	Hypothetical protein (cpp47)
<i>p11601MD_20</i>	93	20	Hypothetical protein
<i>p11601MD_21</i>	798	23	Hypothetical protein (cpp46)
<i>p11601MD_22</i>	765	28	Hypothetical protein
<i>p11601MD_23</i>	438	26	Cag pathogenicity island protein (cpp44)
<i>p11601MD_24</i>	1812	30	Type IV secretion system protein, TraG
<i>p11601MD_25</i>	993	32	Type IV secretion system protein, DNA transfer ATPase, VirB11
<i>p11601MD_26</i>	1173	32	Type IV secretion system protein, VirB10 (cmgB10)
<i>p11601MD_27</i>	888	27	Type IV secretion system protein, VirB9
<i>p11601MD_28</i>	663	27	Type IV secretion system protein, VirB8
<i>p11601MD_29</i>	168	26	Hypothetical protein
<i>p11601MD_30</i>	999	36	Type IV secretory pathway, VirB6
<i>p11601MD_31</i>	972	31	Type IV secretory pathway, VirB5
<i>p11601MD_32</i>	276	24	Translation initiation factor, IF-2
<i>P11601MD_33</i>	426	33	Single-stranded DNA-binding protein
<i>p11601MD_34</i>	666	30	Hypothetical protein
<i>p11601MD_35</i>	558	30	Regulatory protein, Rha family
<i>P11601MD_36</i>	2769	29	Type IV secretory pathway, VirB4
<i>p11601MD_37</i>	264	35	Type IV secretory pathway, VirB2
<i>p11601MD_38</i>	324	26	Hypothetical protein
<i>p11601MD_39</i>	378	27	Virulence-associated protein D, VapD
<i>p11601MD_40</i>	615	26	Resolvase
<i>p11601MD_41</i>	1794	27	DNA repair and recombination protein RadB
<i>p11601MD_42</i>	87	30	Toxin-antitoxin system
<i>p11601MD_43</i>	219	30	Hypothetical protein
<i>p11601MD_44</i>	186	18	Hypothetical protein
<i>p11601MD_45</i>	258	20	Dehydrogenase
<i>p11601MD_46</i>	99	30	Conjugal transfer protein
<i>p11601MD_47</i>	264	32	Hypothetical protein
<i>p11601MD_48</i>	1227	29	DNA primase (cpp22)
<i>p11601MD_49</i>	654	23	Hypothetical protein (cpp21)
<i>p11601MD_50</i>	552	21	NTPase
<i>p11601MD_51</i>	282	19	Hypothetical protein

¹Gray highlight indicates unusually high GC content

The plasmid harbors a well-defined resistance gene region that includes *tet(O)* and, in close vicinity, *aphA-3*, mediating kanamycin resistance (Fig. 1). Immediately downstream of each of these two resistance genes we identified an ORF encoding a hypothetical protein (Fig. 1). These four ORFs have a markedly higher GC content (40-48%) than the remainder of the plasmid ORFs (average, 26.6%), the total p11601MD sequence (29%) and the average (30-33%) described for *C. jejuni* and *C. coli* chromosomal ORFeomes (Walker *et al.*, 1986 and Nuijten *et al.*, 1999) (Table 3), suggesting acquisition of the resistance gene cassette from other source(s) via horizontal gene transfer. Several genes putatively involved in conjugative transfer were identified in the plasmid (Table 3) suggesting potential for horizontal gene transfer of the antibiotic resistance genes via conjugation.

Fig. 1. Resistance gene region of p11601MD. Tetracycline *tet(O)* and kanamycin (*aphA-3*) resistance genes of p11601MD (top) are represented by black arrows with their respective high GC-content flanking ORFs *p11601MD_14* and *p11601MD_12*, respectively (Table 3), represented by gray arrows. Arrow directions indicate ORF orientation. Regions of homology with pN29710-1 (bottom) are represented with diagonal lines and shaded in light gray.

Hypothetical protein *p11601MD_14* in the *tet(O)*–*aphA-3* region of p11601MD appears to have been disrupted with a larger, unrelated sequence in pN29710-1 which includes several resistance determinants, i.e. *sat4*, *aadE*, *hph* and *aad9* encoding aminoglycoside-modifying enzymes (Chen *et al.*, 2013).

The *tet(O)* sequence of p11601MD exhibits 99-100% identity with *tet(O)* identified in numerous other *Campylobacter* plasmids, as well as on the chromosome. The *aphA-3* sequence is also found to be highly similar (99-100% identity) to *aphA-3* from several other *Campylobacter* plasmids. For instance, it exhibited 99% identity with *aphA-3* identified by Gibreel *et al.* in *C. jejuni* plasmid pCJG9 (Gibreel *et al.*, 2004) (accession no. AJ490186). However, the accompanying ORFs (designated ORFA and ORFB) associated with a putative transposase (IS607*) upstream of *aphA-3* in pCJG9 (Gibreel *et al.*, 2004) were not detected in p11601MD. In another example, the *aphA-3* of p11601MD had 100% identity to a gene annotated as 3' aminoglycoside-phosphotransferase in plasmid pN29710-1 (55,127 nt) from *C. coli* strain CVM N29710 (Chen *et al.*, 2013) (Fig. 1). Moreover, *tet(O)* in pN29710-1 and p11601MD were highly conserved (99.38% identity). It was noted, however, that ORF *p11601MD_14* (372 nt, annotated as hypothetical protein) between *tet(O)* and *aphA-3* in p11601MD is 100% conserved in pN29710-1 but disrupted by a large antimicrobial resistance cassette (Fig. 1). In pN29710-1, this cassette harbors 10 ORFs, of which at least four encode putative antimicrobial resistance determinants (Fig. 1). Similarly to the p11601MD region encompassing *tet(O)* and *aphA-3*, the corresponding region of pN29710-1 has a higher GC content than the rest of the plasmid, suggesting acquisition from another source via horizontal gene transfer (Chen *et al.*, 2013). These findings suggest that the *tet(O)*–*aphA-3* cassette in

p11601MD represents a minimal antimicrobial resistance locus that can become expanded in other plasmids by insertion of additional genes.

A recent whole genome sequencing-based analysis of 114 *C. jejuni* and *C. coli* isolates for genes mediating resistance to nine different antimicrobials identified several types of plasmid-borne or chromosomal resistance gene organization (Zhao *et al.*, 2015). Interestingly, four of the nine types of resistance gene cassettes, including the two most predominant among the 114 isolates, harbored both *tet(O)* and *aphA-3* (designated *aph(3')-IIIa*, and exhibiting 100% identity with the *aphA-3* of p11601MD and pN29710-1), with variable genomic content between *tet(O)* and *aphA-3*. In the leading category of 34 isolates, this included several additional resistance determinants between *tet(O)* and *aphA-3*, similar to the arrangement of resistance genes in pN29710-1. In the second largest category (11 of the 114 isolates) the resistance gene cassette included only *tet(O)* and *aphA-3* and was thus similar to that of p11601MD, even though it was not known whether these 11 isolates harbored the *tet(O)* - *aphA-3* cassette on plasmids or chromosomally (Zhao *et al.*, 2015).

To determine potential conservation of p11601MD sequences among other turkey-derived strains of *C. jejuni* with resistance to tetracycline and other antibiotics (Table 1), the strains were tested by PCR using primers derived from various p11601MD regions (Table 2). All strains harbored *tet(O)*, and those resistant to both tetracycline and kanamycin yielded the expected PCR products for all tested ORFs (Fig. 2). On the other hand, both of the two tested kanamycin-susceptible strains were PCR-negative for *aphA-3* and also failed to yield a PCR product with primers derived from the *aphA-3-tet(O)* intergenic region (Fig. 2). These findings suggest that the tested turkey-derived strains of *C. jejuni* harbor plasmids with sequence content

conserved with that of p11601MD. Further sequencing will be needed to determine overall homology between p11601MD and plasmids from these other strains.

	Strain														
PCR target	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
T1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T2	■	□	■	■	■	■	■	■	■	■	■	■	■	□	□
T3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T8	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T9	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□
T10	■	□	■	■	■	■	■	■	■	■	■	■	■	□	□

Fig. 2. *C. jejuni* strains tested by PCR with primers derived from a panel of p11601MD ORFs.

Presence and absence of the expected PCR product is represented in black and white squares, respectively. *C. jejuni* strains S1 (11601MD) and S15 (NCTC 11168) were included as positive and negative control, respectively. Strains and PCR targets are listed in Tables 1 and 2, respectively. PCR was done as described in Materials and Methods.

3. 2. Functional genome distribution analysis.

From the 39 plasmids used in the functional genome distribution (FGD) analysis, p11601MD clustered at the protein level in one cluster (Cluster 1) with 22 other plasmids from *C. jejuni* and other *Campylobacter* spp. (Fig.3A). The other plasmids included in the comparison were grouped in a different cluster (Cluster 2), while *C. coli* RM2228 plasmid pCC2228-1 was placed apart from the two clusters (the other plasmid harbored by *C. coli* RM2228, pCC2228-2, was grouped in Cluster 2) (Fig. 3A). Plasmids in Cluster 2 represent a different plasmid family, comprising a group of very small plasmids harboring a replication protein (RepA/B family),

often a bacterial mobilization protein (MobC family) and one or two other, varying ORFs, but no discernible antimicrobial resistance genes. These two clusters reflect differences in the genetic makeup, and likely origin, of the plasmids investigated.

Fig. 3B shows in more detail the homology among the *tet(O)*-harboring plasmids that clustered together with p11601MD. The *tet(O)*-harboring clade within Cluster 1 includes two subclusters, a p11601MD-like subcluster and a subcluster that shares more similarities with *C. jejuni* S3 plasmid pTet, while plasmids pRM5611 and *C. jejuni* ICDCCJ07001 plasmid pTet are less similar, although still associated with the *tet(O)*-harboring clade (Fig. 3B). Outside this *tet(O)*-clade, only two other plasmids were found with *tet(O)*-homologs in Cluster 1: *C. coli* RM2228 (draft) plasmid pCC178.cb and *C. jejuni* plasmid pCG8245. While all of the *tet(O)* clade plasmids in Cluster 1 harbor *tet(O)*, it appears that their genetic differences are not driven by the absence or presence of antimicrobial resistance genes, but are instead the result of differences in ORFs that are largely annotated as hypothetical. Elucidation of the biological roles of these ORFs may represent a valuable source of understanding of plasmid evolution when *Campylobacter* spp. are exposed to varying environments.

While all plasmids in the *tet(O)* clade of Cluster 1 share *tet(O)* and the *virB* TypeIV secretion system gene cluster, major gene insertion/deletion events can be observed immediately up- and downstream of *tet(O)*. In particular, p11601MD_13 (*aphA*-3, kanamycin resistance) was shared only with three other plasmids in Cluster1, namely *C. coli* RM4661 plasmid pRM4661, *C. jejuni* ssp. *jejuni* 002544 plasmid and *C. coli* CVMN29710 plasmid pN297101. Outside the *tet(O)* clade, only two other plasmids were found to harbor Aph-like proteins (both plasmids also feature a *tet(O)* homolog): *C. coli* RM2228 (draft) plasmid pCC178 which harbors a partial ORF with sequence similarity to p11601MD_13 and *C. jejuni* plasmid pCG8245. This plasmid

(pCG8245) harbors an ORF at the same plasmid locus as the corresponding p11601MD_13 ORF that features a nucleotidyltransferase domain associated with kanamycin resistance (KNTase), yet this ORF shares no amino-acid similarity with p11601MD_13. Interestingly, a second kanamycin resistance gene that exhibits 100% amino acid identity to p11601MD_13 was detected elsewhere in pCG8245, approx. 12kb downstream of the KNTase. These findings imply that the kanamycin resistance determinant *aphA-3* is present in only certain *tet(O)*-harboring *Campylobacter* strains and may have been acquired independently multiple times. This is in agreement with our findings shown in [Fig. 2](#), where *aphA-3* and the intergenic region were the only sequences among those tested that were not conserved in all investigated strains. Thus, a relatively large number of unique or partially shared conserved hypothetical ORFs contribute to the differentiation between the two clades in Cluster 1. The identified hypervariable region centered on *tet(O)* may suggest that antimicrobial resistance genes are acquired rapidly and in response to varying lifestyle pressures that *Campylobacter* spp. may encounter. The fact that *aphA-3* seems restricted to *tet(O)*-harboring plasmids may further strengthen the hypothesis of a *tet(O)*-driven hypervariable region that may facilitate the uptake and diversification of additional antimicrobial resistance genes.

B

Fig. 3. A) Full FGD tree, based on ORFeome and amino acid sequences showing two main clusters. The *tet(O)* clade is indicated separately. B) Clustering of *tet(O)*-harboring plasmids most similar to p11601MD (from Cluster 1 in Fig 3A). Grey boxes indicate sub-clusters 1 and 2.

4. Conclusions

p11601MD, a ca. 44 kb plasmid from a turkey-derived *C. jejuni* strain, harbors a resistance gene cassette with determinants for resistance to tetracycline and kanamycin, i.e. *tet(O)* and *aphA-3*, respectively. This cassette may represent a minimal plasmid-associated resistance locus that can become greatly amplified in various *C. jejuni* and *C. coli* strains by acquisition of additional resistance genes within the cassette. The presence of a type IV secretion system may imply that plasmid DNA can be readily mobilized and translocated, possibly rapidly

distributing antibiotic resistance genes across different *C. jejuni* and *C. coli* strains and across different ecosystems. The hypothesized *tet*(O)-associated hypervariable region may represent a novel mechanism for the uptake and diversification of additional antimicrobial resistance genes. Further analysis of plasmid sequences will be needed to accurately elucidate conservation and dissemination of p11601MD-like plasmids among *C. jejuni* and *C. coli* from different animal hosts in North Carolina and elsewhere, and to elucidate the potential roles of such plasmids in antimicrobial resistance and other aspects of the biology of *Campylobacter* spp.

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Nucleotide sequence accession numbers.

The whole-genome sequence assembly and automatic annotation of *C. jejuni* strain 11601MD plasmid (p11601MD) has been deposited in EMBL/GenBank under accession no. KJ646012.

References

- Alfredson DA, Korolik V.** 2007. Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. FEMS Microbiol Lett. 277(2):123-132.
- Altermann E, Klaenhammer TR.** 2003. GAMOLA: a new local solution for sequence annotation and analyzing draft and finished prokaryotic genomes. OMICS 7:161–169.
- Altermann E.** 2012. Tracing lifestyle adaptation in prokaryotic genomes. Front Microbiol 3:48

- Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J.** 2005. ACT: The Artemis comparison tool. *Bioinformatics* **21**:3422–3423.
- Chen Y, Mukherjee S, Hoffmann M, Kotewicz ML, Young S, Abbott J, Luo Y, Davidson MK, Allard M, McDermott P, Zhao S.** 2013. Whole-genome sequencing of gentamicin-resistant *Campylobacter coli* isolated from U.S. retail meats reveals novel plasmid-mediated aminoglycoside resistance genes. *Antimicrob Agents Chemother* **57**:5398–5405.
- Crespo MD, Olson JW, Altermann E, Siletzky RM, Kathariou S.** 2012. Chromosomal *tet*(O)-harboring regions in *Campylobacter coli* isolates from turkeys and swine. *Appl Environ Microbiol* **78**:8488–8491.
- Dasti JI, Groß U, Pohl S, Lugert R, Weig M, Schmidt-Ott R.** 2007. Role of the plasmid-encoded *tet*(O) gene in tetracycline-resistant clinical isolates of *Campylobacter jejuni* and *Campylobacter coli*. *J Med Microbiol* **56**:833–837.
- Gibreel A, Sköld O, Taylor DE.** 2004. Characterization of plasmid-mediated *aphA-3* kanamycin resistance in *Campylobacter jejuni*. *Microb Drug Resist* **10**:98–105.
- Gillespie IA, O'Brien SJ, Frost JA, Adak GK, Horby P, Swan A V, Painter MJ, Neal KR.** 2002. A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. *Emerg Infect Dis* **8**:937–942.
- Gu W, Siletzky RM, Wright S, Islam M, Kathariou S.** 2009. Antimicrobial susceptibility profiles and strain type diversity of *Campylobacter jejuni* isolates from turkeys in eastern North Carolina. *Appl Environ Microbiol* **75**:474–82.
- Kotarski SF, Merriwether TL, Tkalcevic GT, Gemski P.** 1986. Genetic studies of kanamycin resistance in *Campylobacter jejuni*. *Antimicrob Agents Chemother* **22**:225–230.
- Luangtongkum T, Morishita TY, Ison AJ, Huang S, McDermott PF, Zhang Q.** 2006. Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of *Campylobacter* spp. in poultry. *Appl Environ Microbiol* **72**:3600–3607.
- Luangtongkum T, Jeon B, Han J, Plummer P, Logue MC, Qijing Z.** 2009. Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol* **4**:189–200.
- McCrackin MA, Helke KL, Galloway AM, Poole AZ, Salgado CD, Marriott BP.** 2015. Effect of antimicrobial use in agricultural animals on drug-resistant foodborne campylobacteriosis in humans: A systematic literature review. *Crit Rev Food Sci Nutr* **18**:0.
- Nuijten PJM, Bartels C, Bleumink-Pluym NMC, Gaastra W, van der Zeijst BAM.** 1990. Size and physical chromosome map of the *Campylobacter jejuni* chromosome. *Nucleic Acids Res* **18**:6211–6214.

- Pratt A, Korolik V.** 2005. Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. *J Antimicrob Chemother* **55**:452–460.
- Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B.** 2000. Artemis: sequence visualization and annotation. *Bioinformatics* **16**:944–945.
- Sahin O, Kassem II, Shen Z, Lin J, Rajashekara G, Zhang Q.** 2015. *Campylobacter* in poultry: Ecology and potential interventions. *Avian Dis.* **59(2)**:185-200.
- Scallan E, Hoekstra RM, Angulo FJ, Tauxe R V., Widdowson MA, Roy SL, Jones JL, Griffin PM.** 2011. Foodborne illness acquired in the United States — Major pathogens. *Emerg Infect Dis* **17**:1–21.
- Sneath PH, Sokal RR.** 1962. Numerical taxonomy. *Nature* **193**:855–860.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30**:2725–2729.
- Taylor DE, Garner RS, Allant BJ.** 1983. Characterization of tetracycline resistance plasmids from *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob Agents Chemother* **24**:930–935.
- Taylor DE.** 1986. Plasmid-mediated tetracycline resistance in *Campylobacter jejuni*: Expression in *Escherichia coli* and identification of homology with streptococcal class M determinant. *J Bacteriol* **165**:1037–1039.
- Tenover FC, Williams SUE, Gordon KP, Nolan C, James J.** 1985. Survey of plasmids and resistance factors in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob Agents Chemother* **27**:37–41.
- Walker RI, Caldwell MB, Lee EC, Guerry P, Trevor J, Ruiz-palacios GM.** 1986. Pathophysiology of *Campylobacter* enteritis. *Microbiol Rev* **50**:81–94.
- Zhao S, Tyson GH., Chen Y, Li C, Mukherjee S, Young S, Lam C, Folster J. P, Whichard JM, McDermott PF.** 2015. Whole genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in *Campylobacter* spp. *Appl Environ Microbiol* **82(2)**:459-66.