1 **Tetracycline resistance transmission in *Campylobacter* is promoted at temperatures**
2 **resembling the avian reservoir.**

3 Running title: Thermoregulation of plasmid conjugation in *Campylobacter*.

4

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24 **Abstract**

25 *Campylobacter* is the causal agent of campylobacteriosis in humans, a self-limiting
26 gastroenteritis. Campylobacteriosis is a zoonosis, commonly transmitted from
27 contaminated chicken meat by either direct consumption or cross contamination during
28 food manipulation. Presence of plasmids encoding for resistance to antibiotics such as
29 tetracycline is common among *Campylobacter* isolates. In this report, we studied the effect
30 of the temperature in the conjugation frequency of several *tet*(O) carrying plasmids,
31 providing tetracycline resistance to the recipient cells. The conjugation frequency from
32 donor cells carrying three previously characterized plasmids (pCjA13, pCjA9 and pTet) and
33 from two clinical isolates was determined. Two temperatures, 37 and 42 ºC, mimicking the
34 conditions encountered by *C. jejuni* in the human and broiler chicken gastrointestinal tracts,
35 respectively, were assessed. Our results clearly indicate that the conjugation process is
36 promoted at high temperature. Accordingly, the transcriptional expression of some putative
37 conjugative apparatus genes is thermoregulated, being induced at 42 ºC. The two plasmids
38 present in the clinical isolates were sequenced and assembled. Both plasmids are highly
39 related among them and to the pTet plasmid. The high identity of the genes putatively
40 involved in the conjugation process among the plasmids is in agreement with the similar
41 behavior regarding the temperature dependency of the conjugative process. This report
42 suggest that conjugation of plasmids carrying antibiotic resistance genes occurs
43 preferentially at temperatures that resemble the gastrointestinal tract of birds, the main
44 reservoir of *C. jejuni*.

45 Keywords: *Campylobacter*; tetracycline resistance; plasmid conjugation; temperature
46 regulation

47 **Introduction**

48 *Campylobacter* is a motile Gram-negative epsilon proteobacteria. Thermophilic
49 *Campylobacter* species, particularly *C. jejuni* and *C. coli*, are the leading cause of bacterial
50 gastroenteritis in the European Union, United States and Australia (Havelaar et al., 2015).
51 They cause campylobacteriosis in humans, a self-limiting gastroenteritis characterized by
52 watery diarrhea, abdominal pain and fever. Severe cases are associated with
53 immunocompromised patients such as very young people and the elderly. Moreover,
54 infection by *Campylobacter* has been associated with complications such as reactive
55 arthritis and Guillian-Barré syndrome (Kaakoush et al., 2015).
56 Despite the fact that *Campylobacter* has been isolated from environmental samples, such as
57 water and soils, campylobacteriosis is recognized as a zoonosis. *Campylobacter* is quite
58 common among different animal species, although the major reservoir of *C. jejuni* is the
59 gastrointestinal tract of birds, including broiler chicken (Bronowski et al., 2014). The
60 consumption of contaminated chicken meat is the common source of human
61 campylobacteriosis (EFSA, 2017).
62 Genetically, *Campylobacter* is highly variable. Horizontal gene transfer and recombination
63 events mediate the rapid evolution detected among *Campylobacter* isolates, promoting
64 changes in the pathogenic potential, adaptability to different hosts and spread of antibiotic
65 resistance (Wilson et al., 2009; Woodcock et al., 2017). A widespread dissemination
66 mechanism of genetic information in bacteria is plasmid conjugation. The plasmid
67 occurrence in *C. jejuni* is variable, ranging from 20 to 90 % in different reports (Dasti et al.,
68 2007). In *C. jejuni*, the presence of plasmids is directly related to the pathogenic potential,
69 in the case of the virulence plasmid pVir, or to the antimicrobial resistance profile of the
70 recipient cell, in the case of the tetracycline resistance-carrying plasmid pTet (Poly et al.,

71 2005; Zeng et al., 2015). Genes coding for resistance to antibiotics such as tetracycline,
72 kanamycin, gentamycin and streptomycin have been found in plasmids from *C. jejuni*
73 (Gibreel et al., 2004; Dasti et al., 2007; Abril et al., 2010; Chen et al., 2013). Tetracycline
74 resistance is highly prevalent and, although several mechanisms have been elucidated, is
75 primarily mediated by the protein encoded in the *tet*(O) gene (Elhadidy et al., 2018). This
76 gene encodes for a ribosome-binding protein that promotes the release of tetracycline,
77 allowing protein synthesis in the presence of the antibiotic. The *tet*(O) gene can be located
78 in both, the chromosome and/or in extrachromosomal elements such as plasmids (Gibreel et
79 al., 2004; Pratt and Korolik, 2005). Consistently, tetracycline resistance can be spread by
80 plasmid conjugation (Pratt and Korolik, 2005; Dasti et al., 2007; Luangtongkum et al.,
81 2009).

82 To know the optimal conditions for plasmid conjugation is pivotal to establish efficient
83 strategies for the control of antibiotic resistance spread. In this report, the conjugation
84 frequency of several *tet*(O) carrying plasmids has been assessed at two temperatures, 37
85 and 42 ºC, mimicking the conditions encountered by *C. jejuni* in the human and broiler
86 gastrointestinal tracts, respectively. The results indicate that the conjugation process is
87 promoted at high temperature. Consistent with the differential conjugation frequency
88 described, transcriptional expression of several putative conjugation-related genes is
89 thermoregulated, being induced at 42 ºC as compared to 37 ºC. Our data indicates that
90 conjugation of plasmids carrying antibiotic resistance genes occurs preferentially at
91 temperatures that resemble the gastrointestinal tract of birds, the main reservoir of *C. jejuni*.

92 **Methods**

93 **Bacterial strains, plasmids and growth conditions.**

94 The *C. jejuni* strains used in this work were isolated from human patients suffering from
95 campylobacteriosis. The following strains carrying tetracycline-resistant (Tc^R) plasmids
96 were used as donor cells during mating experiments. Strains A9 and A13 carry the Tc^R
97 plasmids pCjA9 and pCjA13, respectively (Schmidt-Ott et al., 2005). Strain 81-176 carries
98 the plasmids pVir and pTet. The pTet plasmid confers resistance to tetracycline (Poly et al.,
99 2005). The H32 and H61 clinical isolates (Iglesias-Torrens et al., 2018) are characterized to
100 be Tc^R and carrying plasmids. In addition to tetracycline, H32 is resistant to ciprofloxacin
101 and ampicillin, and H61 is resistant to ciprofloxacin and nalidixic acid.

102 The A3 and A3S *C. jejuni* strains were used as recipient strains during mating experiments.
103 The A3 strain is nalidixic acid resistant, tetracycline-susceptible and does not carry any
104 plasmid (Schmidt-Ott et al., 2005). The A3S strain is a streptomycin-resistant derivative of
105 the A3 strain obtained after culturing in presence of increased concentrations of streptomycin.
106 A3S was only used as recipient strain in mating experiments with H32 and H61 strains.

107 The strain H40, previously isolated by our research group (Iglesias-Torrens et al., 2018), was
108 used as negative control for *tet*(O) gene presence during hybridization assays. H40 is
109 susceptible to all antibiotic tested and carries a plasmid. All *C. jejuni* strains were grown on
110 Columbia blood agar base (CBA, Oxoid) supplemented with 5% of defibrinated sheep blood
111 (Oxoid). When required, culture media was supplemented with nalidixic acid (Nal),
112 tetracycline (Tc) and streptomycin (Sm) at 50, 20 and 15 µg/ml, respectively. CBA plates
113 were incubated for 48 hours at either 37 or 42 ºC under microaerophilic conditions using
114 CampyGen™ atmosphere generation system (Oxoid).

115 **Mating experiments**

116 Mating experiments were performed essentially as earlier described (Schmidt-Ott et al.,
117 2005). Donor and recipient strains were grown on selective CBA plates for 48 hours at either
118 37 or 42 ºC. Bacterial cells were collected in phosphate-buffered saline (PBS) supplemented
119 with $MgCl_2$ (100 µM) and the OD_{550} of the cell suspension was normalized to 1.5. Equivalent
120 volumes (50 µl) of recipient and donor cells suspensions were mixed in the presence of
121 DNAse I (100 U/ml) (Roche), to avoid natural transformation events during the assay.
122 Aliquots of 15 µl were spotted on CBA plates supplemented with DNAse I (100 U/ml) and
123 incubated at either 37 or 42 ºC for the indicated times. Cells were recovered in PBS, serially
124 diluted and spread on CBA plates supplemented with the required antibiotics for the selection
125 of both donor and transconjugant cells. Control mating experiments with only donor or
126 recipient cells were included in all experiments. Plates were incubated for 48 h at 42 ºC and
127 the frequency of conjugation was calculated as the number of transconjugants per donor cell.
128 We show the results of at least three independent experiments in a scatter dot plot graphic
129 with the average. Plasmid isolation, restriction profile characterization and strain genotyping
130 by detection of the *wlaN* and *flaA* genes were used to confirm transconjugants selection.

131 **DNA techniques**

132 Genomic and plasmid DNA isolation was extracted by standard procedures using
133 InstaGene™ Matrix (Bio-Rad) and E.Z.N.A.® Plasmid DNA kit (Omega Bio-tek),
134 respectively. Bacterial cultures were grown at 42 ºC for 48 h on CBA plates. *Bgl*II restriction
135 pattern was used to characterize the isolated plasmids. PCR amplification was performed
136 with PCR MasterMix (2x) (Thermo Scientific™). All the primers used are described in Table
137 1.

138 **RNA isolation and RT-PCR assays.**

139 Total RNA was purified from *C. jejuni* cultures grown for 48 h on CBA plates at either 37 or
140 42 ºC. Bacterial cell suspensions in PBS with RNA Protect Bacteria Reagent (Qiagen) were
141 normalized to an OD_{550} of 1.5 and total RNA was isolated using the RNeasy Minikit (Qiagen)
142 following supplier indications.
143 Illustra Ready-to-Go RT-PCR beads (GE Healthcare) were used to perform one step RT-
144 PCR to monitor the expression of *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11* genes. 16S rRNA
145 was used as the internal control. For each specific gene, the amount of template used during
146 PCR amplification was defined by performing saturation curves with increasing amounts of
147 total cDNA to determine the interval of lineal increase in the relative amount of RT-PCR
148 product and total RNA. The relative amount of amplified DNA was determined after 2 %
149 agarose gel electrophoresis using the Image Lab software (Bio-Rad). All the primers used
150 are described in Table 1.

151 **DNA hybridization**

152 DNA samples were subjected to separation by electrophoresis, DNA was transferred and UV
153 light cross-linked onto positively charged nylon membrane by standard methods (Sambrook
154 Fritsch, E.F., Maniatis, T., 1989). Specific digoxigenin-labeled probes for *tet*(O) gene were
155 obtained by PCR with the primer pair *tet*(O)F - *tet*(O)R and the PCR DIG Probe Synthesis
156 kit (Roche). Southern blot hybridization was carried out under high stringency conditions
157 according to the manufacturer's instructions.

158 **Genome sequencing, plasmid assembly and alignment**

159 Whole genome sequencing of the *C. jejuni* isolates H32 and H61 was performed. The DNA
160 extraction was carried out with the Wizard DNA-Purification kit (Promega). Genomic
161 libraries and sequencing were performed in Life Sequencing
162 (http://www.lifesequencing.com/) using the Illumina NextSeq platform and Nextyera XT

163 150PE/TrueSeq DNA kit for library preparation. The pCjH32 and pCjH61 plasmids were
164 assembled from the trimmed reads with SPAdes (Bankevich et al., 2012) v3.13.0 with the
165 plasmid option in addition to default parameters. For pCjH32, a single contig of length 44,740
166 bp was assembled. For pCjH61, three contigs of lengths 31,173 bp, 13,377 bp and 112 bp
167 were assembled. As they were overlapping, we manually assembled them into one contig of
168 44,466 bp. The origin and orientation of both plasmid sequences were adjusted to coincide
169 with that of pTet (AY394561.1).
170 We annotated the assembled plasmids with Prokka (Seemann, 2014) v1.12. To compare the
171 assembled plasmids with the reference plasmid pTet, pairwise BLAST (Altschul et al.,
172 1990) alignments were performed (blastn e-value cutoff of 10^{-5}) and visualized with
173 EasyFig v2.2.2 (Sullivan et al., 2011). Additional pairwise comparisons were made with
174 Mummer v3.22 (Kurtz et al., 2004) in order to determine percent identity. To assign all the
175 genes to their corresponding ortholog in the other plasmids, we performed an all versus all
176 Blastp with all the protein sequences. Then, we selected the hits with more than 75%
177 identity and covering at least an 80% of the sequence length and produce table 2. The gene
178 names were inherited from the pTet reference transcript annotation.

179 **Results**

180 **The conjugation of several *tet*(O) carrying plasmids is promoted at 42 ºC.**

181 To study conjugation of plasmids carrying the *tet*(O) determinant, conferring resistance to
182 tetracycline, the *C. jeuni* A13 strain was initially used as donor strain. A13 carries the 41.9
183 Kb plasmid pCjA13, described as the prototype of the *mob* plasmids, a major subgroup of
184 *tet*(O)-carrying conjugative plasmids in *C. jejuni* (Schmidt-Ott et al., 2005).
185 The mating time required for optimal detection of transconjugants was determined for the
186 donor strain A13 (Tc^R, Nal^S), and the recipient strain A3 (Tc^S, Nal^R). Bacterial cultures and
187 mating assays were incubated at 42 ºC and the conjugation frequency of pCjA13 after
188 increasing mating times was determined (Fig. 1A). Transconjugants were detected after 2.5
189 hours of mating incubation and the conjugation frequency was constant after prolonged
190 incubation (between 2.5 and 5 hours). Colonies selected (Tc^R, Nal^R) were confirmed as
191 transconjugants by *Bgl*II digestion profile of the plasmid DNA and genotyping by PCR
192 detection of the chromosomal *wlaN* gene, since the A3 recipient strain, but not the A13
193 donor strain, carries the *wlaN* gene (Fig. 1B). Therefore, transconjugants clones are
194 characterized by being Nal^R, $wlaN^+$ and carrying the pCjA3 plasmid (Tc^R). From these
195 results, an arbitrary mating incubation time of 4 hours was chosen for all conjugation
196 assays. In a previous report, similar plasmid transfer kinetics were described for the closely
197 related plasmid, pCC31 (Batchelor et al., 2004).
198 *C. jejuni* is commonly found colonizing the gastrointestinal tract of different birds, being
199 the consumption of contaminated poultry meat the most common infection transmission
200 route to humans. The temperature of the gastrointestinal tract of birds and humans is
201 different, being higher in birds (Card et al., 2017). To study the effect of temperature in the
202 conjugation of plasmids carrying the *tet*(O) determinant, the conjugation frequency of

203 pCjA13 was monitored at two temperatures, 42 and 37 ºC, resembling the broiler cecal and
204 the human gut temperatures. Remarkably, pCjA13 plasmid transfer occurs more efficiently
205 at 42 ºC. The conjugation frequency is over 13-fold higher at 42 ºC as compared to 37 ºC
206 (Fig. 1C), suggesting that pCjA13 conjugation can be promoted during broiler colonization.
207 With the same samples used to determine the conjugation frequency, experiments were
208 performed to monitor if the growth temperature (37 and 42 ºC) may affect growth kinetics
209 in such way that could justify the increase in the number of transconjugants detected at
210 42ºC. The concentration of recipient cells at time 0 (starting mating mixtures) and after 4h
211 mating incubation was calculated for mating mixtures incubated at both 37 and 42 ºC
212 (Table S1). The data obtained clearly demonstrate that the bacterial growth during the 4h
213 mating incubation is only slightly promoted (1.3-fold) at 42 ºC as compared at 37 ºC,
214 whereas conjugation frequency is promoted over 13-fold at the highest temperature.
215 Overall, these data rule out that the detection of higher conjugation frequency results from a
216 promoted growth at 42 ºC.
217 The temperature-dependent conjugation of two more previously characterized *tet(O)*
218 carrying plasmids, pCjA9 and pTet, was also determined. Plasmid pCjA9 (40.5 Kb)
219 belongs also to the *mob* plasmids subgroup, although remarkable differences with pCjA13
220 plasmid were reported at the nucleotide sequence level (Schmidt-Ott et al., 2005). The pTet
221 plasmid (45.2 Kb) present in the 81-176 *C. jejuni* strain has been extensively studied
222 (Batchelor et al., 2004; Poly et al., 2005). Conjugation of pCjA9 and pTet to the A3 strain
223 was efficiently detected using the same experimental layout as described above (Fig. 2A). It
224 should be pointed out that strain 81-176 carries two plasmids (pVir and pTet) (Poly et al.,
225 2005), being the *tet*(O) carrying plasmid - pTet - the one selected during our conjugation
226 assay as determined by plasmid restriction analyses (Fig. 2A and (Bacon et al., 2000)). Our

227 results indicate that conjugation of both pCjA9 and pTet is also temperature dependent.

228 Again, temperatures mimicking broiler gut (42 ºC) promotes conjugation as compared with

229 human gut temperature (37 ºC). In this case, more than 8- and 6-fold stimulation was

230 detected for pCjA9 and pTet, respectively (Fig. 2B).

231 Two unrelated *C. jejuni* isolates from patients suffering gastroenteritis, H32 and H61

232 (Iglesias-Torrens et al., 2018), which are tetracycline resistant and carry plasmid DNA,

233 were tested. The plasmid content from both isolates showed a distinct *Bgl*II restriction

234 pattern (Fig. 2C). The presence of a *tet*(O) gene within pCjH32 and pCjH61 was confirmed

235 by Southern hybridization using a *tet*(O) specific probe (Fig. 2D). Mating experiments were

236 performed with H32 and H61 strains as donor strains and A3S, a Sm^R derivative of A3, as

237 recipient strain. Plasmid transfer was efficiently detected. In these experiments, since *wlaN*

238 did not allow discriminating between strain H32 and the transconjugants, detection of the

239 *flaA* gene was used for transconjugant genotyping (Fig. 2C). Our results let us conclude

240 that both *tet*(O) carrying plasmids, pCjH32 and pCjH61, are conjugative. The conjugation

241 frequency of these plasmids was monitored at 37 and 42 ºC and plasmid transfer was also

242 temperature dependent, being 3.5- and 14.5-fold induced at 42 ºC as compared to 37 ºC for

243 pCjH32 and pCjH61, respectively (Fig. 2E).

244 **Transcriptional expression of plasmid transfer-related genes.**

245 To further characterize the observed difference in conjugation frequency, the transcriptional

246 expression pattern of genes putatively involved in plasmid transfer at both assayed

247 temperatures was determined by semi quantitative RT-PCR. Several genes encoding

248 putative T4SS components, named *cmg* (*Campylobacter* mating genes), have been

249 identified in the pTet plasmid for its high homology with genes related with plasmid

250 conjugation from the pVT745 plasmid of *Actinobacillus actinomycetemcomitans* (Batchelor

251 et al., 2004; Poly et al., 2005). Using the pTet annotated sequence (AY394561.1), primers
252 were designed to PCR-amplify fragments of the ORF *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11*.
253 These ORFs presumably code for the major subunit of the conjugative pilus, the pilus-tip
254 adhesion protein, a protein of the inner membrane complex and an ATPase, respectively. It
255 has been described that the plasmids pCjA13, pCjA9 and pTet are somehow related since
256 they share homology among several *cmg* genes (Batchelor et al., 2004; Schmidt-Ott et al.,
257 2005). Accordingly, the designed primers amplify DNA fragments of similar size in
258 genomic samples of strains carrying pTet, pCjA13 and pCjA9. Moreover, similar PCR
259 fragments were also detected in samples of strains carrying plasmids pCjH32 and pCjH61
260 (Fig. 3A), suggesting that this two uncharacterized plasmids are related with the other
261 *tet*(O) carrying plasmids used in this study and that their conjugative apparatus are
262 conserved to some extent (Batchelor et al., 2004; Friis et al., 2007).
263 Total RNA from A13 strain, carrying the pCjA13 plasmid, grown at either 37 or 42 ºC was
264 isolated. Same culture conditions as for conjugation assays were used. The mRNA levels of
265 the above-indicated genes were monitored using RT-PCR (Fig. 3B). Consistent with the
266 increased conjugation frequency detected at 42 ºC, an upregulation in the mRNA levels for
267 the four genes analyzed - *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11* - was detected at high
268 temperature (approximately 3.6-, 4.8-, 9.6- and 7.5-fold, respectively). These results
269 strongly suggest the existence of regulatory pathways that adjust the expression of plasmid-
270 borne genes putatively involved in plasmid transfer in response to temperature changes.
271 This thermoregulation has apparently an impact in the conjugation frequency and may
272 promote plasmid transfer when bacteria are present in the broiler gastrointestinal tract.
273 **Plasmids pCjH32 and pCjH61 are highly similar to the pTet plasmid.**

274 The plasmids from the clinical isolates H32 and H61 were sequenced. pCjH32 and pCjH61
275 have a size of 44.7 and 44.4 Kb and a GC content of 29.62 and 28.34 %, respectively. The
276 annotation revealed 50 and 52 ORFs, being coding sequence approximately 92.3 and 91.8
277 % for pCjH32 and pCjH61, respectively. The tetracycline resistance transferred by these
278 plasmids is encoded in canonical *tetO* genes identical to the pTet plasmid *tetO* gene, with
279 only 1 and 8 nucleotides difference for pCjH32 and pCjH61, respectively. Alignment of the
280 genomes with Mummer shows that pCjH32 has an identity of 95.98% with 3.72%
281 unaligned with respect to pTet, while pCjH61 has an identity of 98.33% with 8.14%
282 unaligned with respect to pTet. A graphical overview based on BLAST alignments is
283 shown in Fig. 4.
284 Putative plasmid conjugation related genes were detected in both pCjH32 and pCjH61
285 plasmids using PCR amplification and primers designed according to the pTet described
286 sequence (Fig. 3). In the pTet plasmid and the closely related pCC31 plasmid, genes
287 putatively coding for T4SS and DNA transfer functions were identified by homology with
288 the T4SS of the pVT745 from *Actinobacillus actinomycetemcomitans* (Batchellor et al.,
289 2004). CmgB2 (pilin) and CmgB5 (minor pilus component) were predicted to be involved
290 in pilus biogenesis. CmgB6 (protein with five transmembrane domains), CmgB7 and
291 Cpp44 (lipoproteins), CmgB9 (periplasmic protein), CmgB8 and CmgB10 (transmembrane
292 proteins) were predicted to form the trans-envelope pore complex. Moreover, three
293 ATPases associated with the T4SS (CmgB4, CmgB11 and CmgD4), a nickase (Cpp17), a
294 DNA primase (Cpp22), a DNA helicase (Cpp26) and a ssDNA binding protein (Cpp34)
295 were predicted to be encoded in these plasmids. All these genes are also found in the two
296 sequenced plasmids, pCjH32 and pCjH61, sharing a high degree of homology at the DNA

297 sequence level and the predicted encoded proteins (Table 2). The homology of all proteins

298 encoded in pCjH32 and pCjH61 among them and to pTet is detailed in Table S2.

299 Batchelor et al, (2004) anticipated the location of the *oriT* in the pTet plasmid between the

300 ORFs *cpp18* and *cpp19*. The proposed sequence contains inverted DNA repeats

301 surrounding a conserved nic site motif ATCCTG as found in other *oriT* sites. Sequence

302 alignment of the equivalent plasmid locations reveals the presence of the nic site motif and

303 a high degree of conservation in the surrounding sequences in the plasmids pCjH32 and

304 pCjH61 when compared to the pTet and the closely related pCC31 plasmids (Table 3).

305

306

307 **Discussion**

308 *Campylobacter* is a highly ubiquitous bacteria, being part of the commensal microbiota of
309 numerous animal species and being isolated from distinct environmental niches (Thépault
310 et al., 2017). The most common transmission route of *Campylobacter* to humans is the
311 consumption of chicken meat. The use of antibiotics as prophylactic and growth promoters
312 for livestock is forbidden in the EU since 2006, however, in many countries these practices
313 are still in use (Maron et al., 2013). One of the antibiotics extensively used in food animal
314 production during the last decades is tetracycline (Fairchild et al., 2005). Accordingly,
315 resistance to tetracycline is one of the most common reported antibiotic resistance among
316 *C. jejuni* isolates (Wieczorek and Osek, 2013; Iglesias-Torrens et al., 2018). These data
317 suggest that the extensive use of this antimicrobial has importantly promoted the spread of
318 *tet*(O), the main tetracycline resistant genetic determinant, among circulating strains of *C.*
319 *jejuni* (Landers et al., 2012). The fact that in many countries the use of tetracyclines have
320 been restricted during food production for the last years, but tetracycline resistance is still
321 highly prevalent, suggest that the *tet*(O) gene remains highly stable in the *Campylobacter*
322 genome (Friis et al., 2007).

323 To know the specific environmental and physiological conditions that promote plasmid
324 transfer is relevant to develop strategies aiming to contain the spread of plasmid borne
325 functions such as antibiotic resistance. Moreover, it should be noted that several reports
326 suggest that the acquisition of plasmids carrying antibiotic resistance genes can be
327 associated with an increase in the virulence of the pathogenic bacteria (Schroeder et al.,
328 2017). In this report we characterized the temperature dependency of the conjugation of
329 plasmids carrying the *tet*(O) gene in *C. jejuni*. We showed that the frequency of conjugation
330 is significantly stimulated at 42 ºC as compared to 37 ºC. In a previous report, it was

331 described that no significant differences were observed in the transfer of Tc^R plasmids of *C.*
332 *jejuni* at 42 and 37 ºC (Taylor et al., 1981). We cannot rule out that the Tc^R plasmid used in
333 these experiments has a differential response to the temperature as compared to the ones
334 used in our report. Nevertheless, it should be noted that in Taylor et al report, the mating
335 mixtures were incubated for 24-48 hours in contrast to the 4 hour incubation used in this
336 report. This difference in the experimental setup may also explain the discrepancies in the
337 results obtained. The stimulation of conjugation at 42 ºC was observed with several *tet*(O)
338 carrying plasmids: pCjA13, pCjA9 and pTet, previously reported to be related by carrying
339 homologous plasmid transfer genes (Batchelor et al., 2004; Schmidt-Ott et al., 2005); and
340 the previously uncharacterized pCjH32 and pCjH61 plasmids from clinical isolates.
341 Remarkably, PCR detection of putative plasmid transfer genes, using pTet specific primers,
342 indicates that all plasmids tested are related. Interestingly, a previous report described
343 unsuccessful transfer of the pTet plasmid by conjugation after several attempts (Bacon et
344 al., 2000). It should be point out that the mating assays were performed at 37 ºC,
345 temperature that is suboptimal for pTet conjugation, as shown in this report. Our data
346 suggest that plasmid transfer might be promoted within the avian reservoir rather that
347 during the transit through the human gastrointestinal tract. *In vivo* experiments previously
348 showed efficient conjugation of *tet*(O) carrying plasmids within chicken gastrointestinal
349 tract (Avrain et al., 2004).
350 Expression studies with four putative conjugation-related genes *cmgB2*, *cmgB5*, *cmgB8* and
351 *cmgB1*, let us conclude that its expression is thermoregulated. Remarkably, the four genes
352 analyzed were higher expressed at high temperature providing a rational justification to the
353 temperature-dependent conjugation of these plasmids.

354 The temperature dependent regulation of plasmid conjugation through transcriptional
355 modulation of genes involved in the synthesis of the conjugative apparatus has been
356 previously reported for the IncHI1 plasmid R27 (Alonso et al., 2005; Forns et al., 2005).
357 The conjugation of plasmids IncHI1, associated with the spread of antibiotic resistances
358 among enterobacteria, is stringently thermoregulated (Taylor and Levine, 1980). IncHI1
359 plasmids conjugation is promoted at low temperature suggesting that plasmid transfer
360 occurs during the transit of the bacteria outside the mammal hosts (Maher and Taylor,
361 1993). Our data clearly indicate that the plasmids present in two Tc^R clinical isolates are
362 highly related among them and to the pTet plasmid. Accordingly, with the similar response
363 to temperature shown by the conjugation of the plasmid studied, the high identity is
364 extensive to all genes presumably involved in plasmid conjugation. The similarity is not
365 restricted to the ORFs; it is also high among the surrounding non-coding sequences,
366 indicating similar promoter sequences that control the expression of the conjugation related
367 genes in the different plasmids. In this report, we described that in *C. jejuni* the conjugation
368 of *tet*(O) carrying plasmids is stimulated at higher temperature indicating that antibiotic
369 spread occurs more efficiently within the avian reservoir.
370

371 **Acknowledgment**

372 Dr. Andreas Zautner from Universitätsmedizin Göttingen for kindly providing the A3, A9
373 and A13 strains. Dr. Patricia Guerry from the U.S. Naval Medical Research Center for
374 kindly providing the 81-176 strain.

375 **Funding sources**

376 This work was supported by the Spanish Ministry of Economy and Competitiveness [grant
377 AGL2013-45339R], Spanish Ministry of Science, Innovation and Universities [grant
378 PGC2018-096958-B-I00] and the Catalonian government [grant 2017SGR499]. PG was
379 recipient of an ADR fellowship of the University of Barcelona.

380 **Competing of interest statement**

381 Declaration of interest: none.

382

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517

518 **Table 1. Oligonucleotides used in this report.**
519
520

Gene	Source	Forward (Fw) and Reverse (Rv) primers	Reference
cmgB2	pTet plasmid (AY394561.1)	Fw 5' GCTGGTGGTATTGATAAAGT 3' Rv 5' TGATCCAAAAATAACGCCAC 3'	This study
cmgB5	pTet plasmid (AY394561.1)	Fw 5' AGCCATAGGGAAATCTCATC 3' Rv 5' GGAATACCAGCACTAAATGC 3'	This study
cmgB8	pTet plasmid (AY394561.1)	Fw 5' GACAATACTACAGGAATGGT 3' Rv 5' TCTCTTGCATTATCACCTTC 3'	This study
cmgB11	pTet plasmid (AY394561.1)	Fw 5' GAAATCTGCTATAACGGCGA 3' Rv 5' AGCGAGTTTTGCTTGGCTTT 3'	This study
tet(O)	pTet plasmid (AY394561.1)	Fw 5' GCGTTTTGTTTATGTGCG 3' Rv 5' ATGGACAACCCGACAGAAG 3'	Bacon *et al*., 2000
16S	LMG 9217 (AF550626.1)	Fw 5' GGATGACACTTTTCGGAG 3' Rv 5' CCTCCACTCTAGACTATC 3'	This study
wlaN	NCTC 11168 (AL111168.1)	Fw 5' TGCTGGGTATACAAAGGTTGTG 3' Rv 5' AGGTCCATTACCGCATACCA 3'	Koolman *et al*., 2015
flaA	81-176 (AF345999.1)	Fw 5' AATAAAAATGCTGATAAAACAGGTG 3' Rv 5' TACCGAACCAATGTCTGCTCTGATT 3'	Datta et al., 2003

521

522 **Table 2. Predicted conjugation related coding regions on pCjH32 and pCjH61 plasmids and the closest relationships to the**
523 **functional homologues in pTet.**

		% identity DNA* - % identity (% similarity) proteins**		Length (aa)
Gene	**Predicted function**	**pCjH32**	**pCjH61**	**pTet / pCjH32 / pCjH61**
cpp17	Nickase	98.3 - 97.0 (99.4)	97.9 - 96.8 (99.4)	462 / 462 / 462
cpp22	Primase	95.6 - 95.1 (98.8)	99.7 - 99.8 (100)	408 / 408 / 408
cpp26	DNA helicase	96.5 - 97.5 (99.7)	99.9 - 100 (100)	597 / 597 / 597
cmgB2 (*cpp30*)	Pilin	92.4 - 92.0 (97.7)	92.0 -92.0 (98.9)	87 / 87 / 87
cmgB3/4 (*cpp31*)	ATPase	97.9 - 98.7 (99.8)	96.5 - 98.2 (99.8)	883 / 922 / 922
cpp34	ssDNA binding protein	97.9 - 97.2 (99.3)	97.6 - 97.2 (99.3)	141 / 140 / 140
cmgB5 (*cpp36*)	Pilus minor component	94.6 - 89.2 (97.5)	97.4 - 93.2 (98.1)	323 / 329 / 329
cmgB6 (*cpp37*)	Transmembrane protein (TEPC)	85.5 - 84.9 (96.4)	86.7 - 85.3 (96.4)	281 / 331 / 331
cmgB7 (*cpp38*)	Lipoprotein (TEPC)	95.8 - 94.5 (98.2)	99.4 - 100 (100)	55 / 54 / 55
cmgB8 (*cpp39*)	Transmembrane protein (TEPC)	84.9 - 86.2 (97.2)	99.5 - 100 (100)	220 / 219 / 220
cmgB9 (*cpp40*)	Periplasmic protein (TEPC)	89.8 - 89.2 (97.0)	97.7 - 98.0 (100)	295 / 296 / 295
cmgB10 (*cpp41*)	Transmembrane protein (TEPC)	85.2 - 86.1 (94.9)	99.6 -99.5 (100)	398 / 392 / 391
cmgB11 (*cpp42*)	ATPase	91.4 - 96.0 (98.8)	99.9 - 100 (100)	348 / 335 / 330
cmgD4 (*cpp43*)	ATPase	93.9 - 94.9 (98.8)	99.7 - 99.8 (100)	603 / 603 / 603
cpp44	Lipoprotein (TEPC)	99.5 - 99.3 (100)	99.5 - 99.3 (100)	145 / 145 / 145

524 TEPC: Trans-envelope porus complex. * % of DNA identity determined by BLAST (NCBI, NIH). ** % of protein identity and
525 similarity determined by LALIGN (EXPASI, SIB)

526 **Table 3. Alignment of the putative *oriT* in plasmid pTet, pCjH32 and pCjH61.** The *nic*

527 site motif ATCCTG is indicated (grey shadow).

528

Plasmid	Sequence
pCjH32	TTTGAGAAATAAAAGGCTATCCTG**T**AAT---C----ATTAAA
pCjH61	TTTGAGAAATAAAAGGCTATCCTGCAAT---C----ATTAAA
pTet	TTTGAGAAATAAAAGGCTATCCTGCAATTATCAATTATTAAA
pCC31	TTTGAGAAATAAAAGGCTATCCTGCAAT---C----ATTAAA

529
530

531 **Figure captions**

532 **Figure 1**. Transfer of the conjugative tetracycline resistance plasmid pCjA13 in *C. jejuni*.
533 A. Conjugation frequency of plasmid pCjA13 to A3 recipient cells after different mating
534 incubation times. Cultures and mating mixtures were incubated at 42 ºC. B. Genotyping of
535 recipient (R), donor (D) and transconjugants (T): *Bgl*II restriction profile of plasmid DNA
536 (upper panel) and *wlaN* PCR amplification (lower panel). C. Conjugation frequency of
537 plasmid pCjA13 to A3 recipient cells at 37 and 42 ºC. Both cultures and mating mixtures
538 were incubated at the indicated temperatures. Significance was tested by an impaired two-
539 tailed t-test. Statistical significance is indicated by *** p<0.001.

540 **Figure 2**. Temperature dependent transfer of conjugative tetracycline resistance plasmids.
541 A. Upper panels, *Bgl*II restriction profile of pCjA9 and pTet plasmids from recipient (R),
542 donor (D) and transconjugant (T) cells. R strain was A3, D strains were A9 and 81-176 for
543 pCjA9 and pTet, respectively. In lower panels, *wlaN* PCR amplification. Notice that the 81-
544 176 strain carries two plasmids, pVir and the Tc^R plasmid pTet, whereas in its derivative
545 transconjugant only the pTet plasmid was detected. B. Conjugation frequency of pCAj9 and
546 pTet plasmids at 37 and 42 ºC using donor and recipient cells as in A. C. Genotyping of R,
547 D and T cells. R strain was A3S, D strains were H32 and H61 for pCjH32 and pCjH61,
548 respectively. *Bgl*II restriction profile of pCjH32 and pCjH61 plasmids (upper panels), *wlaN*
549 PCR amplification (middle panels) and *flaA* PCR amplification (lower panel). D. Southern
550 hybridization using a digoxygenin labeled *tet*(O) specific probe. Plasmid samples from the
551 clinical isolates H32 and H61 and the control strains A3(pTet) (positive control) and H40
552 (negative control) were analyzed. H40 is tetracycline susceptible (Iglesias-Torrens et al.,
553 2018). Left panel, plasmid samples after ethidium bromide staining (EtBr). Right panel,
554 *tet*(O) detection in the plasmid samples. E. Conjugation frequency of pCjH32 and pCjH61

555 plasmids at 37 and 42 ºC. Donor and recipient cells as in C. In B and E, both cultures and
556 mating mixtures were incubated at the indicated temperatures. Significance was tested by
557 an impaired two-tailed t-test. Statistical significance is indicated by * $p<0.05$, ** $p<0.01$,
558 *** $p<0.001$.

559 **Figure 3**. Transcription analysis by semi-quantitative RT-PCR of the selected genes coding
560 for putative proteins of the conjugative apparatus. A. PCR- amplification using specific
561 primers for the *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11* homologous genes using genomic
562 DNA from the strains 81-176, A13, A9 and A3, A3 (pTet), H32 and H61. B.
563 Transcriptional expression of the conjugation related genes was monitored by semi
564 quantitative RT-PCR. The RNA was extracted from cultures of the A13 strain grown at 37
565 and 42 ºC. RNA16S amplification was included as a control to confirm that equivalent
566 amounts of template were used. RNA samples from three independent cultures were tested
567 by RT-PCR obtaining similar results. The images correspond to gels from a representative
568 experiment.

569 **Figure 4.** Sequence comparison plasmids pCjH32, pCjH61 and pTet (reference plasmid,
570 AY394561.1). Nucleotide BLAST percent identity is color-coded according to the legend.
571 Genes predicted with Prokka in pCjH32 and pCjH61 and named according to their
572 correspondence to genes in pTet are shown on the top and bottom, respectively. Genes that
573 are not present in pTet but in the other two plasmids have been labeled with their putative
574 function when possible.

575