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

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

Cuevas-Ferrando, E.[1*], Guirado, P.[1*], Miró, E.[2], Iglesias-Torrens, Y.[2,3], Navarro, F.[2,3], Alioto, T. S.[4,5], Gómez-Garrido, J.[4], Madrid, C.[1], Balsalobre, C.[1]

[1]Department of Genetics, Microbiology and Statistics. School of Biology. University of Barcelona. Av. Diagonal, 643. 08028. Barcelona (Spain).

[2]Hospital de la Santa Creu i Sant Pau and Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), Barcelona, Spain.

[3]Departament de Genètica i Microbiologia. Universitat Autònoma de Barcelona, Barcelona, Spain.

[4]CNAG-CRG, Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology (BIST), Baldiri i Reixac 4, 08028 Barcelona, Spain

[5]Universitat Pompeu Fabra (UPF), Barcelona, Spain

*Both authors equally contributed.

Corresponding authors:

Dra. Cristina Madrid. Department of Genetics, Microbiology and Statistics. University of Barcelona. Av. Diagonal 643, 08028 Barcelona, Spain. E-mail: cmadrid@ub.edu, tel: +34934039382, fax: +34934034639.

Dr. Carlos Balsalobre. Department of Genetics, Microbiology and Statistics. University of Barcelona. Av. Diagonal 643, 08028 Barcelona, Spain. E-mail: cbalsalobre@ub.edu, tel: +34934034622, fax: +34934034639.

Abstract

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

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

Introduction

Campylobacter is a motile Gram-negative epsilon proteobacteria. Thermophilic *Campylobacter* species, particularly *C. jejuni* and *C. coli*, are the leading cause of bacterial gastroenteritis in the European Union, United States and Australia (Havelaar et al., 2015). They cause campylobacteriosis in humans, a self-limiting gastroenteritis characterized by watery diarrhea, abdominal pain and fever. Severe cases are associated with immunocompromised patients such as very young people and the elderly. Moreover, infection by *Campylobacter* has been associated with complications such as reactive arthritis and Guillian-Barré syndrome (Kaakoush et al., 2015).

Despite the fact that *Campylobacter* has been isolated from environmental samples, such as water and soils, campylobacteriosis is recognized as a zoonosis. *Campylobacter* is quite common among different animal species, although the major reservoir of *C. jejuni* is the gastrointestinal tract of birds, including broiler chicken (Bronowski et al., 2014). The consumption of contaminated chicken meat is the common source of human campylobacteriosis (EFSA, 2017).

Genetically, *Campylobacter* is highly variable. Horizontal gene transfer and recombination events mediate the rapid evolution detected among *Campylobacter* isolates, promoting changes in the pathogenic potential, adaptability to different hosts and spread of antibiotic resistance (Wilson et al., 2009; Woodcock et al., 2017). A widespread dissemination mechanism of genetic information in bacteria is plasmid conjugation. The plasmid occurrence in *C. jejuni* is variable, ranging from 20 to 90 % in different reports (Dasti et al., 2007). In *C. jejuni*, the presence of plasmids is directly related to the pathogenic potential, in the case of the virulence plasmid pVir, or to the antimicrobial resistance profile of the recipient cell, in the case of the tetracycline resistance-carrying plasmid pTet (Poly et al.,

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

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

Methods

Bacterial strains, plasmids and growth conditions.

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

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

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

Mating experiments

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

DNA techniques

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

RNA isolation and RT-PCR assays.

Total RNA was purified from *C. jejuni* cultures grown for 48 h on CBA plates at either 37 or 42 ºC. Bacterial cell suspensions in PBS with RNA Protect Bacteria Reagent (Qiagen) were normalized to an OD_{550} of 1.5 and total RNA was isolated using the RNeasy Minikit (Qiagen) following supplier indications.

Illustra Ready-to-Go RT-PCR beads (GE Healthcare) were used to perform one step RT-PCR to monitor the expression of *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11* genes. 16S rRNA was used as the internal control. For each specific gene, the amount of template used during PCR amplification was defined by performing saturation curves with increasing amounts of total cDNA to determine the interval of lineal increase in the relative amount of RT-PCR product and total RNA. The relative amount of amplified DNA was determined after 2 % agarose gel electrophoresis using the Image Lab software (Bio-Rad). All the primers used are described in Table 1.

DNA hybridization

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

Genome sequencing, plasmid assembly and alignment

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

150PE/TrueSeq DNA kit for library preparation. The pCjH32 and pCjH61 plasmids were assembled from the trimmed reads with SPAdes (Bankevich et al., 2012) v3.13.0 with the plasmid option in addition to default parameters. For pCjH32, a single contig of length 44,740 bp was assembled. For pCjH61, three contigs of lengths 31,173 bp, 13,377 bp and 112 bp were assembled. As they were overlapping, we manually assembled them into one contig of 44,466 bp. The origin and orientation of both plasmid sequences were adjusted to coincide with that of pTet (AY394561.1).

We annotated the assembled plasmids with Prokka (Seemann, 2014) v1.12. To compare the assembled plasmids with the reference plasmid pTet, pairwise BLAST (Altschul et al., 1990) alignments were performed (blastn e-value cutoff of 10^{-5}) and visualized with EasyFig v2.2.2 (Sullivan et al., 2011). Additional pairwise comparisons were made with Mummer v3.22 (Kurtz et al., 2004) in order to determine percent identity. To assign all the genes to their corresponding ortholog in the other plasmids, we performed an all versus all Blastp with all the protein sequences. Then, we selected the hits with more than 75% identity and covering at least an 80% of the sequence length and produce table 2. The gene names were inherited from the pTet reference transcript annotation.

Results

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

To study conjugation of plasmids carrying the *tet*(O) determinant, conferring resistance to tetracycline, the *C. jeuni* A13 strain was initially used as donor strain. A13 carries the 41.9 Kb plasmid pCjA13, described as the prototype of the *mob* plasmids, a major subgroup of *tet*(O)-carrying conjugative plasmids in *C. jejuni* (Schmidt-Ott et al., 2005).

The mating time required for optimal detection of transconjugants was determined for the donor strain A13 (Tc^R, Nal^S), and the recipient strain A3 (Tc^S, Nal^R). Bacterial cultures and mating assays were incubated at 42 ºC and the conjugation frequency of pCjA13 after increasing mating times was determined (Fig. 1A). Transconjugants were detected after 2.5 hours of mating incubation and the conjugation frequency was constant after prolonged incubation (between 2.5 and 5 hours). Colonies selected (Tc^R, Nal^R) were confirmed as transconjugants by *Bgl*II digestion profile of the plasmid DNA and genotyping by PCR detection of the chromosomal *wlaN* gene, since the A3 recipient strain, but not the A13 donor strain, carries the *wlaN* gene (Fig. 1B). Therefore, transconjugants clones are characterized by being Nal^R, *wlaN*$^+$ and carrying the pCjA3 plasmid (Tc^R). From these results, an arbitrary mating incubation time of 4 hours was chosen for all conjugation assays. In a previous report, similar plasmid transfer kinetics were described for the closely related plasmid, pCC31 (Batchelor et al., 2004).

C. jejuni is commonly found colonizing the gastrointestinal tract of different birds, being the consumption of contaminated poultry meat the most common infection transmission route to humans. The temperature of the gastrointestinal tract of birds and humans is different, being higher in birds (Card et al., 2017). To study the effect of temperature in the conjugation of plasmids carrying the *tet*(O) determinant, the conjugation frequency of

pCjA13 was monitored at two temperatures, 42 and 37 ºC, resembling the broiler cecal and the human gut temperatures. Remarkably, pCjA13 plasmid transfer occurs more efficiently at 42 ºC. The conjugation frequency is over 13-fold higher at 42 ºC as compared to 37 ºC (Fig. 1C), suggesting that pCjA13 conjugation can be promoted during broiler colonization. With the same samples used to determine the conjugation frequency, experiments were performed to monitor if the growth temperature (37 and 42 ºC) may affect growth kinetics in such way that could justify the increase in the number of transconjugants detected at 42ºC. The concentration of recipient cells at time 0 (starting mating mixtures) and after 4h mating incubation was calculated for mating mixtures incubated at both 37 and 42 ºC (Table S1). The data obtained clearly demonstrate that the bacterial growth during the 4h mating incubation is only slightly promoted (1.3-fold) at 42 ºC as compared at 37 ºC, whereas conjugation frequency is promoted over 13-fold at the highest temperature. Overall, these data rule out that the detection of higher conjugation frequency results from a promoted growth at 42 ºC.

The temperature-dependent conjugation of two more previously characterized *tet(O)* carrying plasmids, pCjA9 and pTet, was also determined. Plasmid pCjA9 (40.5 Kb) belongs also to the *mob* plasmids subgroup, although remarkable differences with pCjA13 plasmid were reported at the nucleotide sequence level (Schmidt-Ott et al., 2005). The pTet plasmid (45.2 Kb) present in the 81-176 *C. jejuni* strain has been extensively studied (Batchelor et al., 2004; Poly et al., 2005). Conjugation of pCjA9 and pTet to the A3 strain was efficiently detected using the same experimental layout as described above (Fig. 2A). It should be pointed out that strain 81-176 carries two plasmids (pVir and pTet) (Poly et al., 2005), being the *tet*(O) carrying plasmid - pTet - the one selected during our conjugation assay as determined by plasmid restriction analyses (Fig. 2A and (Bacon et al., 2000)). Our

results indicate that conjugation of both pCjA9 and pTet is also temperature dependent. Again, temperatures mimicking broiler gut (42 ºC) promotes conjugation as compared with human gut temperature (37 ºC). In this case, more than 8- and 6-fold stimulation was detected for pCjA9 and pTet, respectively (Fig. 2B).

Two unrelated *C. jejuni* isolates from patients suffering gastroenteritis, H32 and H61 (Iglesias-Torrens et al., 2018), which are tetracycline resistant and carry plasmid DNA, were tested. The plasmid content from both isolates showed a distinct *Bgl*II restriction pattern (Fig. 2C). The presence of a *tet*(O) gene within pCjH32 and pCjH61 was confirmed by Southern hybridization using a *tet*(O) specific probe (Fig. 2D). Mating experiments were performed with H32 and H61 strains as donor strains and A3S, a Sm^R derivative of A3, as recipient strain. Plasmid transfer was efficiently detected. In these experiments, since *wlaN* did not allow discriminating between strain H32 and the transconjugants, detection of the *flaA* gene was used for transconjugant genotyping (Fig. 2C). Our results let us conclude that both *tet*(O) carrying plasmids, pCjH32 and pCjH61, are conjugative. The conjugation frequency of these plasmids was monitored at 37 and 42 ºC and plasmid transfer was also temperature dependent, being 3.5- and 14.5-fold induced at 42 ºC as compared to 37 ºC for pCjH32 and pCjH61, respectively (Fig. 2E).

Transcriptional expression of plasmid transfer-related genes.

To further characterize the observed difference in conjugation frequency, the transcriptional expression pattern of genes putatively involved in plasmid transfer at both assayed temperatures was determined by semi quantitative RT-PCR. Several genes encoding putative T4SS components, named *cmg* (*Campylobacter* mating genes), have been identified in the pTet plasmid for its high homology with genes related with plasmid conjugation from the pVT745 plasmid of *Actinobacillus actinomycetemcomitans* (Batchelor

et al., 2004; Poly et al., 2005). Using the pTet annotated sequence (AY394561.1), primers were designed to PCR-amplify fragments of the ORF *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11*. These ORFs presumably code for the major subunit of the conjugative pilus, the pilus-tip adhesion protein, a protein of the inner membrane complex and an ATPase, respectively. It has been described that the plasmids pCjA13, pCjA9 and pTet are somehow related since they share homology among several *cmg* genes (Batchelor et al., 2004; Schmidt-Ott et al., 2005). Accordingly, the designed primers amplify DNA fragments of similar size in genomic samples of strains carrying pTet, pCjA13 and pCjA9. Moreover, similar PCR fragments were also detected in samples of strains carrying plasmids pCjH32 and pCjH61 (Fig. 3A), suggesting that this two uncharacterized plasmids are related with the other *tet*(O) carrying plasmids used in this study and that their conjugative apparatus are conserved to some extent (Batchelor et al., 2004; Friis et al., 2007).

Total RNA from A13 strain, carrying the pCjA13 plasmid, grown at either 37 or 42 ºC was isolated. Same culture conditions as for conjugation assays were used. The mRNA levels of the above-indicated genes were monitored using RT-PCR (Fig. 3B). Consistent with the increased conjugation frequency detected at 42 ºC, an upregulation in the mRNA levels for the four genes analyzed - *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11* - was detected at high temperature (approximately 3.6-, 4.8-, 9.6- and 7.5-fold, respectively). These results strongly suggest the existence of regulatory pathways that adjust the expression of plasmid-borne genes putatively involved in plasmid transfer in response to temperature changes. This thermoregulation has apparently an impact in the conjugation frequency and may promote plasmid transfer when bacteria are present in the broiler gastrointestinal tract.

Plasmids pCjH32 and pCjH61 are highly similar to the pTet plasmid.

The plasmids from the clinical isolates H32 and H61 were sequenced. pCjH32 and pCjH61 have a size of 44.7 and 44.4 Kb and a GC content of 29.62 and 28.34 %, respectively. The annotation revealed 50 and 52 ORFs, being coding sequence approximately 92.3 and 91.8 % for pCjH32 and pCjH61, respectively. The tetracycline resistance transferred by these plasmids is encoded in canonical *tetO* genes identical to the pTet plasmid *tetO* gene, with only 1 and 8 nucleotides difference for pCjH32 and pCjH61, respectively. Alignment of the genomes with Mummer shows that pCjH32 has an identity of 95.98% with 3.72% unaligned with respect to pTet, while pCjH61 has an identity of 98.33% with 8.14% unaligned with respect to pTet. A graphical overview based on BLAST alignments is shown in Fig. 4.

Putative plasmid conjugation related genes were detected in both pCjH32 and pCjH61 plasmids using PCR amplification and primers designed according to the pTet described sequence (Fig. 3). In the pTet plasmid and the closely related pCC31 plasmid, genes putatively coding for T4SS and DNA transfer functions were identified by homology with the T4SS of the pVT745 from *Actinobacillus actinomycetemcomitans* (Batchellor et al., 2004). CmgB2 (pilin) and CmgB5 (minor pilus component) were predicted to be involved in pilus biogenesis. CmgB6 (protein with five transmembrane domains), CmgB7 and Cpp44 (lipoproteins), CmgB9 (periplasmic protein), CmgB8 and CmgB10 (transmembrane proteins) were predicted to form the trans-envelope pore complex. Moreover, three ATPases associated with the T4SS (CmgB4, CmgB11 and CmgD4), a nickase (Cpp17), a DNA primase (Cpp22), a DNA helicase (Cpp26) and a ssDNA binding protein (Cpp34) were predicted to be encoded in these plasmids. All these genes are also found in the two sequenced plasmids, pCjH32 and pCjH61, sharing a high degree of homology at the DNA

sequence level and the predicted encoded proteins (Table 2). The homology of all proteins encoded in pCjH32 and pCjH61 among them and to pTet is detailed in Table S2. Batchelor et al, (2004) anticipated the location of the *oriT* in the pTet plasmid between the ORFs *cpp18* and *cpp19*. The proposed sequence contains inverted DNA repeats surrounding a conserved nic site motif ATCCTG as found in other *oriT* sites. Sequence alignment of the equivalent plasmid locations reveals the presence of the nic site motif and a high degree of conservation in the surrounding sequences in the plasmids pCjH32 and pCjH61 when compared to the pTet and the closely related pCC31 plasmids (Table 3).

Discussion

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

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

described that no significant differences were observed in the transfer of Tc^R plasmids of *C. jejuni* at 42 and 37 ºC (Taylor et al., 1981). We cannot rule out that the Tc^R plasmid used in these experiments has a differential response to the temperature as compared to the ones used in our report. Nevertheless, it should be noted that in Taylor et al report, the mating mixtures were incubated for 24-48 hours in contrast to the 4 hour incubation used in this report. This difference in the experimental setup may also explain the discrepancies in the results obtained. The stimulation of conjugation at 42 ºC was observed with several *tet*(O) carrying plasmids: pCjA13, pCjA9 and pTet, previously reported to be related by carrying homologous plasmid transfer genes (Batchelor et al., 2004; Schmidt-Ott et al., 2005); and the previously uncharacterized pCjH32 and pCjH61 plasmids from clinical isolates. Remarkably, PCR detection of putative plasmid transfer genes, using pTet specific primers, indicates that all plasmids tested are related. Interestingly, a previous report described unsuccessful transfer of the pTet plasmid by conjugation after several attempts (Bacon et al., 2000). It should be point out that the mating assays were performed at 37 ºC, temperature that is suboptimal for pTet conjugation, as shown in this report. Our data suggest that plasmid transfer might be promoted within the avian reservoir rather that during the transit through the human gastrointestinal tract. *In vivo* experiments previously showed efficient conjugation of *tet*(O) carrying plasmids within chicken gastrointestinal tract (Avrain et al., 2004).

Expression studies with four putative conjugation-related genes *cmgB2*, *cmgB5*, *cmgB8* and *cmgB1*, let us conclude that its expression is thermoregulated. Remarkably, the four genes analyzed were higher expressed at high temperature providing a rational justification to the temperature-dependent conjugation of these plasmids.

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

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Competing of interest statement

Declaration of interest: none.

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Table 1. Oligonucleotides used in this report.

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

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

Gene	Predicted function	% identity DNA* - % identity (% similarity) proteins**		Length (aa)
		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

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

Table 3. Alignment of the putative *oriT* in plasmid pTet, pCjH32 and pCjH61. The *nic* site motif ATCCTG is indicated (grey shadow).

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

Figure captions

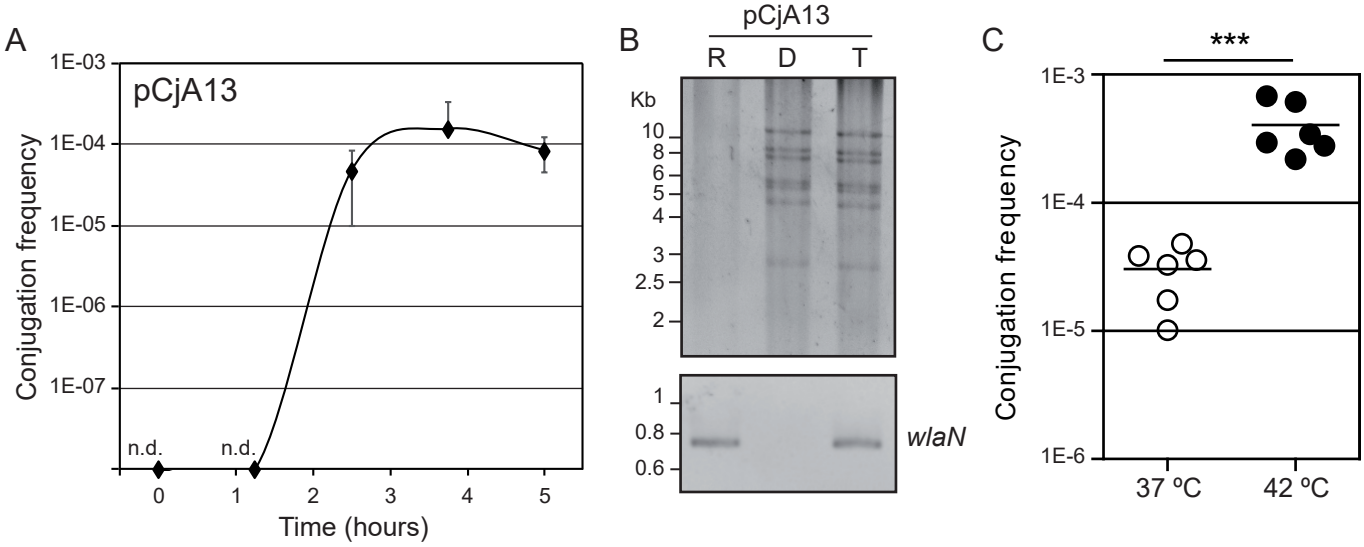
Figure 1. Transfer of the conjugative tetracycline resistance plasmid pCjA13 in *C. jejuni*. A. Conjugation frequency of plasmid pCjA13 to A3 recipient cells after different mating incubation times. Cultures and mating mixtures were incubated at 42 ºC. B. Genotyping of recipient (R), donor (D) and transconjugants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

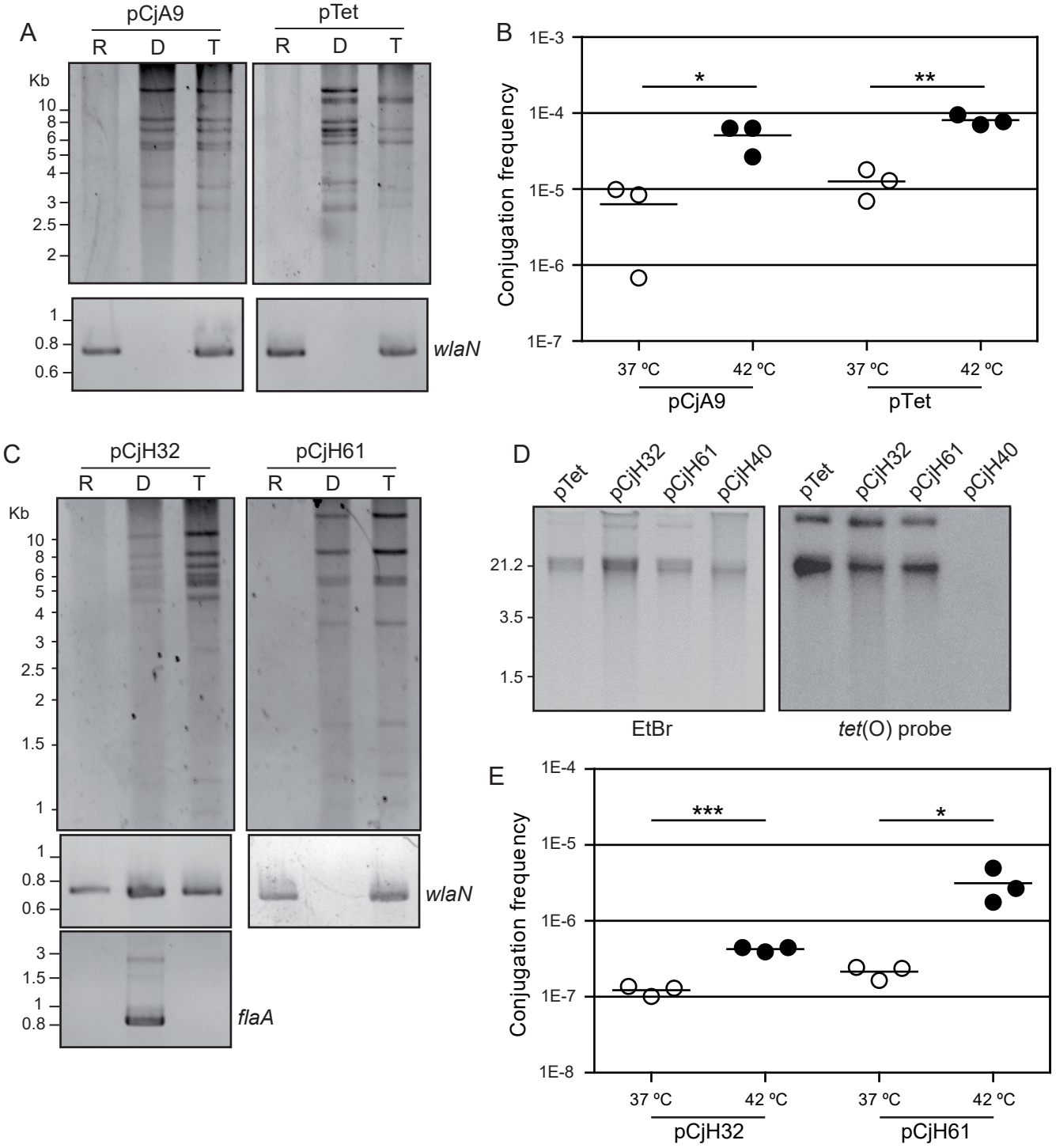
Figure 2. Temperature dependent transfer of conjugative tetracycline resistance plasmids. A. Upper panels, *Bgl*II restriction profile of pCjA9 and pTet plasmids from recipient (R), donor (D) and transconjugant (T) cells. R strain was A3, D strains were A9 and 81-176 for pCjA9 and pTet, respectively.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

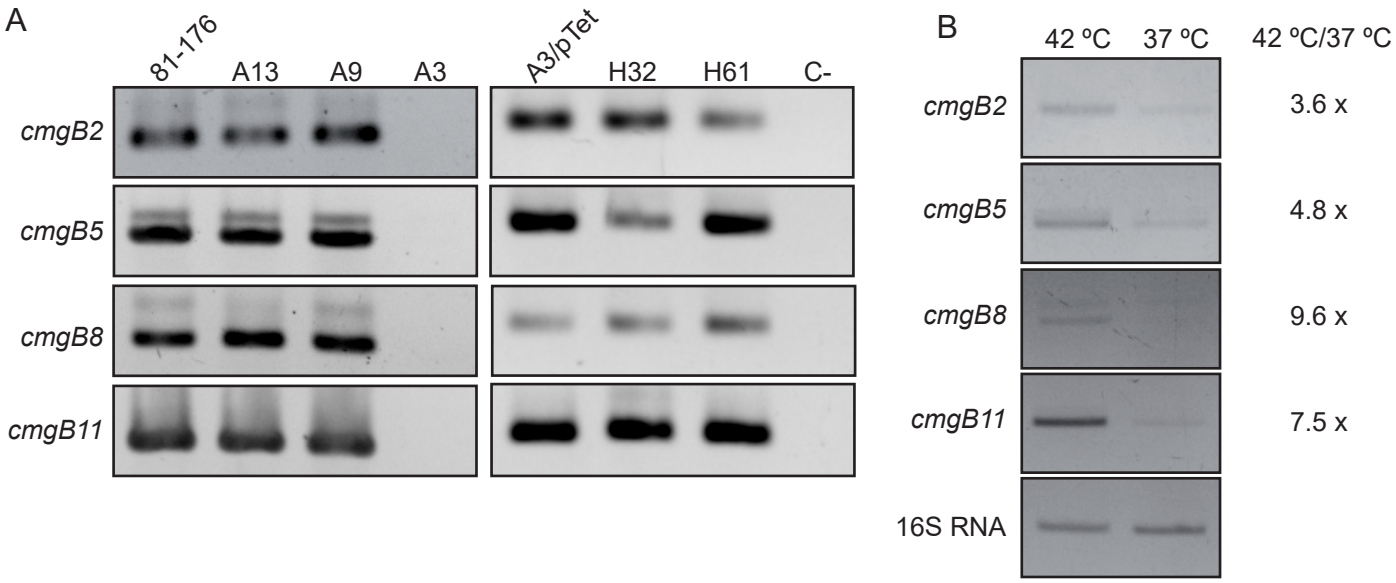
plasmids at 37 and 42 ºC. Donor and recipient cells as in C. In B and E, both cultures and mating mixtures were incubated at the indicated temperatures. Significance was tested by an impaired two-tailed t-test. Statistical significance is indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

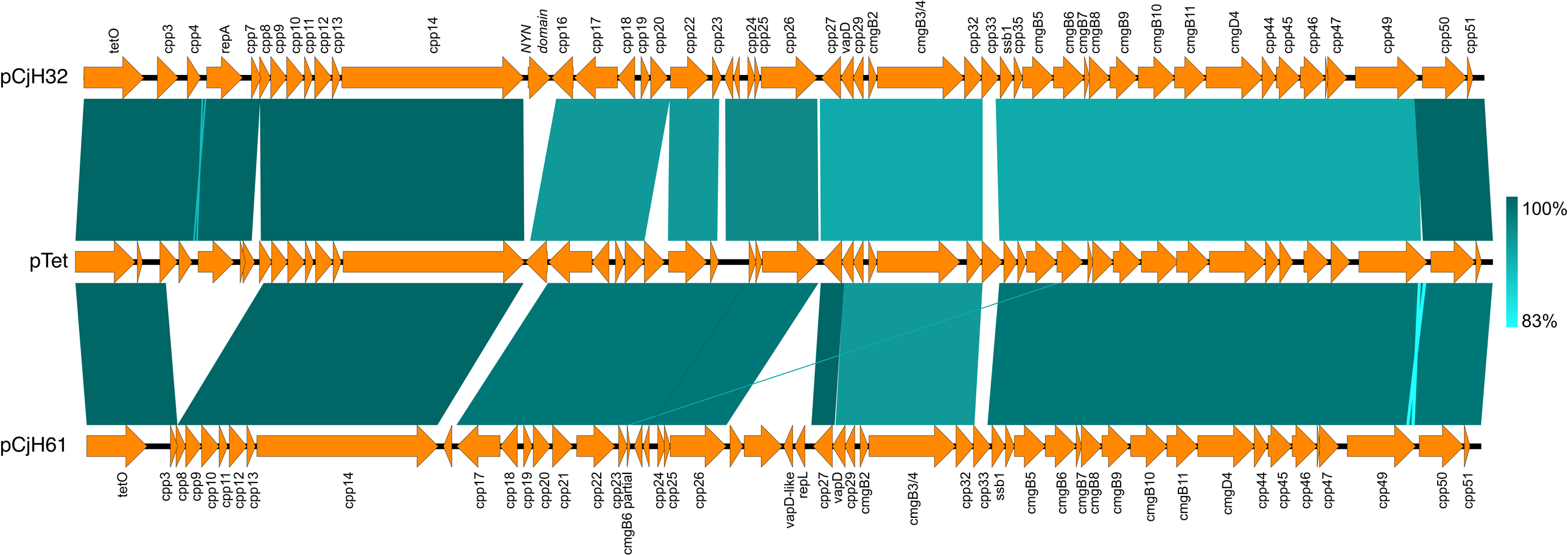
Figure 3. Transcription analysis by semi-quantitative RT-PCR of the selected genes coding for putative proteins of the conjugative apparatus. A. PCR- amplification using specific primers for the *cmgB2*, *cmgB5*, *cmgB8* and *cmgB11* homologous genes using genomic DNA from the strains 81-176, A13, A9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

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


A
pCjA13
Conjugation frequency
1E-03
1E-04
1E-05
1E-06
1E-07
n.d.
n.d.
0
1
2
3
4
5
Time (hours)
B
pCjA13
R
D
T
Kb
10
8
6
5
4
3
2.5
2
1
0.8
0.6
wlaN
C
***
Conjugation frequency
1E-3
1E-4
1E-5
1E-6
37 ºC
42 ºC


A
pCjA9
pTet
R
D
T
Kb
10
8
6
5
4
3
2.5
2
1
0.8
0.6
wlaN
B
Conjugation frequency
1E-3
1E-4
1E-5
1E-6
1E-7
*
**
37 ºC
42 ºC
pCjA9
pTet
C
pCjH32
pCjH61
flaA
D
pTet
pCjH32
pCjH61
pCjH40
21.2
3.5
1.5
EtBr
tet(O) probe
E
1E-4
1E-5
1E-6
1E-7
1E-8
***
pCjH32
pCjH61


A
81-176
A13
A9
A3
A3/pTet
H32
H61
C-
cmgB2
cmgB5
cmgB8
cmgB11
B
42 ºC
37 ºC
42 ºC/37 ºC
cmgB2
3.6 x
cmgB5
4.8 x
cmgB8
9.6 x
cmgB11
7.5 x
16S RNA


pCjH32
pTet
pCjH61
tetO
cpp3
cpp4
repA
cpp7
cpp8
cpp9
cpp10
cpp11
cpp12
cpp13
cpp14
NYN domain
cpp16
cpp17
cpp18
cpp19
cpp20
cpp22
cpp23
cpp24
cpp25
cpp26
cpp27
vapD
cpp29
cmgB2
cmgB3/4
cpp32
cpp33
ssb1
cpp35
cmgB5
cmgB6
cmgB7
cmgB8
cmgB9
cmgB10
cmgB11
cmgD4
cpp44
cpp45
cpp46
cpp47
cpp49
cpp50
cpp51
cpp21
cmgB6 partial
vapD-like
repL
100%
83%

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

Cuevas, E., Guirado, P., Miró, E., Iglesias-Torrens, Y., Navarro, F., Alioto, T. S., Gómez-Garrido, J., Madrid, C., Balsalobre, C.

Supplementary information

	Conjugation frequency av.	Recipient cell growth T4/T0*
37ºC	3.00E-05	2.71±0.6
42ºC	4.04E-04	3.69±0.5
Ratio 42ºC/37ºC	13.3	1.36

Table S1. Conjugation frequency and bacterial growth at 37 versus 42 ºC. The average conjugation frequency of plasmid pCjA13 to A3 recipient cells at 37 and 42 ºC was calculated from six conjugation mixtures (detailed data shown in Fig. 1C). The ratio 42ºC/37ºC indicate that conjugation occurs >10-fold more efficiently at 42 ºC. Recipient cell growth T4/T0 indicate the fold duplication of the recipient cells during the 4 hours incubation of the mating mixtures at the indicated temperatures, the concentration of recipient cells at 4h was divided by the concentration at time 0. The ratio 42ºC/37ºC indicate that bacterial growth is only slightly promoted at 42ºC as compared at 37ºC (1.3 fold). Overall, these data rule out that the detection of higher conjugation frequency results from a promoted growth at 42 ºC.

Table S2. Protein homology between pTet, pCjH32 and pCjH61. The predicted ORFs from plasmid pTet, pCjH32 and pCjH61 plasmids and the percentage of identity among the ORFs for the indicated comparisons are given. To assign all the genes to their corresponding ortholog in the other plasmids, we performed an all versus all Blastp with all the protein sequences. Hits with more than 75% identity and covering at least an 80% of the sequence length were selected. The gene names were inherited from the pTet reference transcript annotation.

				% Identity		
Gene	**pTet ID**	**pCjH32 ID**	**pCjH61 ID**	**pTet/pCjH32**	**pTet/pCjH61**	**pCjH32/pCjH61**
tet(O)	AAR29535.1	AOHAJODB_00001	EOHDCONB_00001	99.8	99.1	98.9
cpp2	AAR29536.1					
cpp3	AAR29537.1					
		AOHAJODB_00002	EOHDCONB_00002			100.0
cpp4	AAR29538.1	AOHAJODB_00003		100.0		
repA	AAR29539.1	AOHAJODB_00004		100.0		
cpp6	AAR29540.1					
cpp7	AAR29541.1	AOHAJODB_00005		100.0		
cpp8	AAR29542.1	AOHAJODB_00006	EOHDCONB_00003	100.0	100.0	100.0
cpp9	AAR29543.1	AOHAJODB_00007	EOHDCONB_00004	100.0	100.0	100.0
cpp10	AAR29544.1	AOHAJODB_00008	EOHDCONB_00005	100.0	100.0	100.0
cpp11	AAR29545.1	AOHAJODB_00009	EOHDCONB_00006	100.0	100.0	100.0
cpp12	AAR29546.1	AOHAJODB_00010	EOHDCONB_00007	100.0	100.0	100.0
cpp13	AAR29547.1	AOHAJODB_00011	EOHDCONB_00008	97.7	97.7	100.0
cpp14	AAR29548.1	AOHAJODB_00012	EOHDCONB_00009	99.4	99.5	99.8
cpp16	AAR29549.1					
		AOHAJODB_0013				
		AOHAJODB_0014				
			EOHDCONB_00010			
cpp17	AAR29550.1	AOHAJODB_00015	EOHDCONB_00011	97.0	96.8	98.1

cpp18	AAR29551.1	AOHAJODB_00016	EOHDCONB_00012	100.0	95.6	95.6
cpp19	AAR29552.1	AOHAJODB_00017	EOHDCONB_00013	96.8	96.8	100.0
cpp20	AAR29553.1	AOHAJODB_00018	EOHDCONB_00014	94.0	100.0	94.0
cpp21	AAR29554.1		EOHDCONB_00015		98.2	
cpp22	AAR29555.1	AOHAJODB_00019	EOHDCONB_00016	95.1	99.8	95.3
cpp23	AAR29556.1	AOHAJODB_00020	EOHDCONB_00017	85.9	88.2	95.7
*	AAR29570.1	AOHAJODB_00036	EOHDCONB_00018		93.1	90.3
		AOHAJODB_00021	EOHDCONB_00019			94.0
		AOHAJODB_00022	EOHDCONB_00020			83.6
cpp24	AAR29557.1	AOHAJODB_00023	EOHDCONB_00021	97.2	100.0	97.2
cpp25	AAR29558.1	AOHAJODB_00024	EOHDCONB_00022	94.6	98.4	92.0
cpp26	AAR29559.1	AOHAJODB_00025	EOHDCONB_00023	97.5	100.0	97.5
			EOHDCONB_00024			
			EOHDCONB_00025			
			EOHDCONB_00026			
			EOHDCONB_00027			
cpp27	AAR29560.1	AOHAJODB_00026	EOHDCONB_00028	93.6	100.0	93.6
vapD	AAR29561.1	AOHAJODB_00027	EOHDCONB_00029	83.7	91.0	85.0
cpp29	AAR29562.1	AOHAJODB_00028	EOHDCONB_00030	99.1	99.1	98.1
cmgB2	AAR29563.1	AOHAJODB_00029	EOHDCONB_00031	92.0	92.0	83.9
cmgB3/4	AAR29564.1	AOHAJODB_00030	EOHDCONB_00032	98.7	98.2	97.2
cpp32	AAR29565.1	AOHAJODB_00031	EOHDCONB_00033	81.5	86.8	84.0
cpp33	AAR29566.1					
		AOHAJODB_00032	EOHDCONB_00034			99.5
ssb1	AAR29567.1	AOHAJODB_00033	EOHDCONB_00035	97.2	97.2	100.0
cpp35	AAR29568.1	AOHAJODB_00034	EOHDCONB_00036	97.8	97.8	100.0
cmgB5	AAR29569.1	AOHAJODB_00035	EOHDCONB_00037	90.0	94.1	95.7

cmgB6	AAR29570.1	AOHAJODB_00036	EOHDCONB_00038	84.9	85.3	95.8
cmgB7	AAR29571.1	AOHAJODB_00037	EOHDCONB_00039	94.5	100.0	94.5
cmgB8	AAR29572.1	AOHAJODB_00038	EOHDCONB_00040	86.2	100.0	86.2
cmgB9	AAR29573.1	AOHAJODB_00039	EOHDCONB_00041	89.2	98.0	90.6
cmgB10	AAR29574.1	AOHAJODB_00040	EOHDCONB_00042	86.1	99.5	85.8
cmgB11	AAR29575.1	AOHAJODB_00041	EOHDCONB_00043	96.0	100.0	96.0
cmgD4	AAR29576.1	AOHAJODB_00042	EOHDCONB_00044	94.9	99.8	94.7
cpp44	AAR29577.1	AOHAJODB_00043	EOHDCONB_00045	99.3	99.3	100.0
cpp45	AAR29578.1	AOHAJODB_00044	EOHDCONB_00046	100.0	100.0	100.0
cpp46	AAR29579.1	AOHAJODB_00045	EOHDCONB_00047	100.0	100.0	100.0
		AOHAJODB_00046	EOHDCONB_00048			100.0
cpp47	AAR29580.1	AOHAJODB_00047	EOHDCONB_00049	100.0	100.0	100.0
cpp49	AAR29581.1	AOHAJODB_00048	EOHDCONB_00050	91.5	99.2	91.2
cpp50	AAR29582.1	AOHAJODB_00049	EOHDCONB_00051	100.0	99.8	99.8
cpp51	AAR29583.1	AOHAJODB_00050	EOHDCONB_00052	100.0	100.0	100.0

*The EOHDCONB_00018 ORF shows a high percentage of identity with *cmgB6* from both pTet and pCJH32. The length of the EOHDCONB_00018 ORF (93 bp) is shorter than *cmgB6* (843 bp) indicating that is the result of a partial translocation.