DNA Sequence Heterogeneity of *Campylobacter jejuni* CJIE4 Prophages and Expression of Prophage Genes

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Abstract

*Campylobacter jejuni* carry temperate bacteriophages that can affect the biology or virulence of the host bacterium. Known effects include genomic rearrangements and resistance to DNA transformation. *C. jejuni* prophage CJIE1 shows sequence variability and variability in the content of morons. Homologs of the CJIE1 prophage enhance both adherence and invasion to cells in culture and increase the expression of a specific subset of bacterial genes. Other *C. jejuni* temperate phages have so far not been well characterized. In this study we describe investigations into the DNA sequence variability and protein expression in a second prophage, CJIE4. CJIE4 sequences were obtained de novo from DNA sequencing of five *C. jejuni* isolates, as well as from whole genome sequences submitted to GenBank by other research groups. These CJIE4 DNA sequences were heterogeneous, with several different insertions/deletions (indels) in different parts of the prophage genome. Two variants of a 3–4 kb region inserted within CJIE4 had different gene content that distinguished two major conserved CJIE4 prophage families. Additional indels were detected throughout the prophage. Detection of proteins in the five isolates characterized in our laboratory in isobaric Tags for Relative and Absolute Quantitation (iTRAQ) experiments indicated that prophage proteins within each of the two large indel variants were expressed during growth of the bacteria on Mueller Hinton agar plates. These proteins included the extracellular DNase associated with resistance to DNA transformation and prophage repressor proteins. Other proteins associated with known or suspected roles in prophage biology were also expressed from CJIE4, including capsid protein, the phage integrase, and MazF, a type II toxin-antitoxin system protein. Together with the results previously obtained for the CJIE1 prophage these results demonstrate that sequence variability and expression of moron genes are both general properties of temperate bacteriophages in *C. jejuni*.

Introduction

Prophages were first demonstrated in *Campylobacter* spp. in the 1960s and 1970s, but it was only in 2003 that whole genome sequencing confirmed their presence in *C. jejuni*, *C. coli*, and *C. lari* [1]. These prophages appear to be structurally diverse and mobile within the genome [2]; a consequence that has been demonstrated to arise from this is changes in PFGE patterns [3]. They can be induced from the bacterial genome by lytic phage predation and thus cause genome rearrangements [4] and they carry genes for extracellular DNases responsible for a dramatic reduction in the frequency of natural transformation [5,6]. We have previously found sequence variability in *Campylobacter jejuni* integrated element 1 (CJIE1) prophages [7,8] that was associated with differences in virulence among isolates. CJIE1 prophages were associated with increased adherence and invasion to INT-407 cells in culture of the prophage-carrying isolates [9], phenotypes associated with differential expression of a subset of bacterial genes and proteins [10]. Like prophages in *Escherichia coli* and *Salmonella enterica* [11], it is possible that *C. jejuni* prophages may carry effectors or other proteins that are virulence factors expressed by the host bacterium.

If prophages of *C. jejuni* were found to play roles similar to those of *E. coli* and *Salmonella*, an understanding of the contribution of these prophages to virulence and host adaptation could be critical for surveillance, source tracking, and control of the subset(s) of *C. jejuni* responsible for human disease. The investigations that form the basis of this report were designed to query whether there was sequence variability in a second *C. jejuni* prophage, CJIE4, and to investigate whether variably carried genes were expressed as proteins and could therefore affect the biology of the host bacterium.

CJIE4 was observed by Fouts et al. to exhibit minimal DNA sequence similarity to CJIE1 from isolate RM1221 and greater similarity to both CJIE2 from *C. jejuni* RM1221 and CLIE1 from *C. lari* RM2100 [1]. CJIE4 prophages were found in 21% of the *C. jejuni* tested and showed rather limited diversity in gene content by comparative genomic analysis using DNA microarrays [2]. Whole genome sequencing of *C. jejuni* strain 414 also demonstrated the presence of a prophage homologous to CJIE4, though less than 75% of the RM1221 CJIE4 genes were present [12]. Similar variability in CJIE4 content was reported by Pittenger et al. [13] in
having an influence on the biology of the host bacterium. With the function or biology of the phages themselves rather than homology to known proteins suggested that most or all of the proteins encoded by conserved prophage genes. However, an analysis of the middle of the genome and several other differentially carried genes throughout. Proteins encoded by genes within both major variants of the large central indel were detected, as were additional proteins encoded by conserved prophage genes. However, an examination of the putative functions of these proteins based on homology to known proteins suggested that most or all of the moron proteins carried by CJIE4 prophages may be associated with the function or biology of the phages themselves rather than having an influence on the biology of the host bacterium.

**Materials and Methods**

**Isolates and growth conditions**

The five isolates used for the study were associated with the investigation into the spring 2000 *Campylobacter* and *E. coli* outbreak in Walkerton, Ontario, Canada [16,17]. All isolates previously tested positive in PCR assays for the presence of three genes from CJIE4 [8]. One of the goals of the study was to assess diversity in CJIE4 prophages; therefore, isolates with different serotypes and isolated in different years were chosen where possible [16]. The Oxford multi-locus sequence types (STs) for the five isolates are: 00-2425, ST 21; 00-0949 and 01-1512, ST 8; 00-6200, ST 806; 00-1597, ST 930.

*C. jejuni* isolates were kept for long-term storage in either 20% skim milk or glycerol peptone water (25% v/v glycerol, 10 g/L peptone) at −80°C. For use, *C. jejuni* isolates with a low passage number were retrieved from storage at 2

**DNA Sequencing and annotation**

Prophages were sequenced by PCR amplification and DNA sequencing of 2–3 kb fragments as outlined previously [7,8]. Sequences were compiled using the SeqMan program in the DNAStar Lasergene 8 package. Open reading frames were initially detected using the Entrez query “viruses [orgn]” (NCBI). Annotations were checked and corrected using GenDB and the annotated prophage genomes were used to create GenBank submissions.

**Accession numbers for DNA sequences submitted to GenBank**

The whole genome sequence of isolate 00-2425 is CP006729. The accession numbers for the CJIE4 prophages from the other four isolates are as follows: 00-0949, KF751793; 00-1597, KF751794; 00-2425, KF751795; 00-6200, KF751796; 01-1512, KF751797.

**Sequences obtained from GenBank**

Full-length sequences of the CJIE4 homologs from isolates 00-0949 and 00-2425 were used to perform a BLASTn search of the nr database in Genbank. The latest date that sequences were checked was June 3, 2013.

Accession numbers for contiguous full length sequences in *C. jejuni* were: RM1221, NC_003912.2; S3, NC_017281.1; 140-16, AIPF010006.1; 1997-4, AIOW01000013.1; 414, ADGM010 00161.8; 84-25, AANT0200001.1. In other cases more than one contig was required to obtain coverage of the homologous prophage; in some cases, contigs containing only one or a few loci were assumed to be part of the larger prophage based on synteny with one or more of the five prophages sequenced for this study. Included were C. jejuni strains: DFVF1099, ADHK1000001.1, ADHK10000040.1; 1997-14, AIPF01000032.1, AIPF0100009.1, AIPF01000012.01, AIPF01000033.1, 51037, NZ_AIPB10000015.1, NZ_AIPB10000046.1, NZ_AIPB100 0089.1, 51494. AINZ01000093.1, AINZ01000066.1, AINZ01000122.1, 87459, AIPF01000015.1, AIE01000029.1, AIE01000086.1. A single C. coli sequence from strain 2548 (AIML01000004.1, AIML01000028.1, AIML01000068.1, AIML01000098.1) was used.

**PCR for verifying the location of the CJIE4 prophage within isolet 01-1512**

All PCR reactions were done using DNA extracted from bacteria using a Gentra Systems PUREGENE DNA Isolation kit (Qiagen) according to the instructions of the manufacturer. PCR reactions were run using reagents from FastStart Taq DNA Polymerase kits (Roche). For each reaction the following parameters were used: final MgCl2 concentrations of 2.0 mM, 0.2 mM of each dNTP, 0.5 μM of each primer, and 2.5 U of FastStart DNA polymerase. PCR reactions were performed using a Perkin Elmer 9700 thermocycler.

PCR primers were developed on the basis of the location of the CJIE4 prophage within the draft whole genome sequence of *C. jejuni* isolate 01-1512, in which the prophage was inserted between genes encoding homologs of CJE0341 (upstream) and Cj0339c (downstream). Within each primer pair, one primer was located within a CJIE4 gene and one was located in the adjacent genomic DNA in order to unambiguously locate the genomic insertion site. Upstream primers were CJIE4uF 5′ GAG ATC TTT TTG CCT TGG GAG TT 3′ located in the homolog of CJE0341 (NCTC11168; CJE0386 in RM1221) and CJIE4uR 5′ TCG ATG ACG TGT GAA CGC TTG AT 3′ located within the homolog of CJIE1418 (RM1221 CJIE4). These primers had an optimal annealing temperature of 50.8°C and produced a 907 bp product. Cycle conditions for the primer pair were: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 50.8°C for 1 min, 72°C for 1 min; final extension at 72°C for 7 min; 4°C until reactions were analyzed.

Primers made to the other end of the CJIE4 prophage were CJIE4dR 5′ TGG GTG TAT GGG CTT GTG GAG TT 3′ located in the intergenic region between homologs of CJE1472 and 1473 (RM1221 homologs) and CJIE4dF 5′ TGG GAG TAA 3′ located within CJ0339 (NCTC11168; CJE0384 in RM1221). This primer pair had an optimal annealing temperature of 48.9°C and produced a 1544 bp amplicon. Cycle conditions for the primer pair were: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 48.9°C for 1 min, 72°C for 2 min; final extension at 72°C for 7 min; 4°C until reactions were analyzed. Reaction products were analyzed by submarine electrophoresis using 1.6% agarose gels and stained with GelRed Nucleic Acid Stain (Cedarlane).
Preparation of iTRAQ-labeled proteins for comparative proteomics analysis

Total cellular proteins were prepared, modified, and labelled with iTRAQ reagents as previously described [10]. Bacteria were recovered after 48 h growth on Mueller-Hinton agar plates with sterile Dulbecco’s PBS, pH 7.0–7.2 ( Gibco, Invitrogen), washed once with PBS, and suspended in sterile high-quality 18 MΩ (MilliQ) water. Acid-washed 212–300 μm glass beads (Sigma-Aldrich Canada Ltd.) were added to the suspension, vortexed, and then boiled for 5 min. Proteins were released from the boiled cells by several rounds of vortexing followed by shaking gently for 5 min on a vortexer fitted with a 12 place Ambion Vortexer Adapter for Genie 2 Vortex Mixer attachment (Applied Biosystems Canada). After centrifugation for 1 min at 3000 rpm (664 g) the protein-containing supernatant was collected into a sterile 15 ml centrifuge tube. Protein preparations were used immediately or stored at −80°C for up to two months. The protein concentration of each preparation was estimated using a Pierce Protein Assay kit (Fisher Scientific) according to the manufacturer’s protocol.

Protein modification and digestion in cartridges was done according to previously published methods [10,18]. Crude protein suspensions containing 100 μg of protein were dried in a Savant DNA120 SpeedVac Concentrator (Fisher Scientific). Proteins were solubilized in 50 μl of freshly made SDS solubilization buffer (4% SDS, 50 mM HEPES buffer pH 8.3, 100 mM DTT) by heating at 95°C for 5 min. Urea Exchange Buffer (UEB; 0038 M urea in 50 mM HEPES, pH 8.3) was added to dilute the protein suspensions, proteins were added to Nanosep 10 K cartridges (VWR International LLC) and the buffer was exchanged with fresh UEB by centrifugation of the protein mix and dilution with fresh UEB. Proteins were then alkylated by adding 100 μl of 50 mM iodoacetamide (IAA Reagent, Sigma-Aldrich) in UEB, shaking for 5 min at RT, and incubation for 20 min without shaking in the dark. After removal of iodoacetamide by exchanging buffer three times with UEB alone, the UEB buffer was exchanged twice with 150 μl of 50 mM HEPES, pH 8.3. DNA was removed by the incubation with 50 μl of Benzonase (Sigma-Aldrich) solution (20 U/μl Benzonase in 42 mM HEPES, pH 8.3 containing 2 mM MgCl₂), shaking at 600 rpm for 2 min at RT and incubation without shaking for 30 min at RT. After this incubation, the cartridge was washed three times with 100 μl of 50 mM HEPES, pH 8.3.

Proteins were digested ON with 5 μl (5 μg) trypsin (Trypsin Gold, mass spectrometry grade, Promega) in the Nanosep 10 K cartridges. Following digestion the tryptic peptides were recovered from the cartridge, dried, and suspended in 30 μl 100 mM HEPES, pH 8.3. iTRAQ labels (AB Sciex Pte Ltd.) were suspended in 100% ethanol, added to the peptide mixtures, and incubated ON at RT. After quenching the reactions with sterile MilliQ water the mixtures were dried and stored at −20°C until use.

Liquid chromatography and mass spectrometry

iTRAQ-labeled tryptic peptide samples (100 μg) were fractionated by high-pH, C18-reversed phase liquid chromatography on a micro-flow Agilent 1100/1200 series system (Agilent Technologies), using a Waters XBridge C18 guard column (10 mm long, 2.1 mm inner diameter, 3.5 μm particles) and a Waters XBridge C18 analytical column (10 cm long, 2.1 mm inner diameter, 3.5 μm particles). Mixed peptides were dried and suspended in LC buffer A (20 mM ammonium formate, pH 10), then resolved by a gradient of LC buffer A and buffer B (20 mM ammonium formate and 90% acetonitrile, pH 10). The gradient started at 3% B from 0–10 min, 8–11% B from 10–17 min; 11–60% B from 17–75 min; 95% B from 75–80 min; and 3% B from 80–170 min at a constant flow rate of 150 μl/min. Fractions were collected across the peptides elution profile (10–75 min), dried and resuspended in 40 μl of nano LC buffer A (2% acetonitrile, 0.1% formic acid).

Each fraction was separately analysed using a nano-flow Easy nLC II (Thermo Fisher Scientific) connected in-line to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) with a nano-electrospray ion source (Thermo Fisher Scientific). The peptide fractions (6 μl) were loaded onto a C18-reversed phase trap column (2 cm long, 100 μm inner diameter, 5 μm particles) with 100% buffer A (2% acetonitrile, 0.1% formic acid) at 4 μl/min for a total volume of 30 μl, and then separated on a C18-reversed phase column (15 cm long, 75 μm inner diameter, 3 μm particles). Both columns were packed in-house with ReproSil-Pur C18-AQ resin (Dr. Maisch). Peptides were eluted using a linear gradient of 2–20% buffer B (98% acetonitrile, 0.1% formic acid) over 120 min at a constant flow rate of 250 nl/min. The total LC/MS/MS runtime was 160 minutes, including the loading, linear gradient, column wash at 95% buffer B, and the equilibration.

Data were acquired using a data-dependent method, dynamically choosing the top 10 abundant precursor ions from each survey scan for isolation in the LTQ and fragmentation by HCD at 45% normalized collision energy. The survey scans were acquired in the Orbitrap over m/z 300–1700 with a target resolution of 60,000 at m/z 400, and the subsequent fragment ion scans were acquired in the Orbitrap over a dynamic m/z range with a target resolution of 7500 at m/z 400. The lower threshold for selecting a precursor ion for fragmentation was 1000 counts. Dynamic exclusion was enabled using a list size of 500 features, a m/z tolerance of 15 ppm, a repeat count of 1, a repeat duration of 30 s, and an exclusion duration of 15 s, with early expiratory disabled.

Data processing

All spectra were processed using Mascot Distiller v2.4.1 (Matrix Science), and database searching was done with Mascot v2.4 (Matrix Science). Searches were performed against an in-house built, non-redundant database consisting of NCBI’s Genome database of bacteria [ftp://ftp.ncbi.nlm.nih.gov/genomes/] and prokaryotic and bacteriophage sequences generated in-house. The decoy database option was selected and the following parameters were used: carbamidomethylation (C) and iTRAQ (K and N-terminus) as fixed modifications, oxidations (M) as a variable modification, fragment ion mass tolerance of 0.5 Da, parent ion tolerance of 10 ppm, and trypsin enzyme with up to 1 missed cleavage. Mascot search results were imported into Scaffold Q+ v4.0 (Proteome Software) and filtered using 1.0% Protein FDR; 2 peptides; 0.1% Peptide FDR.

Results

CJIE4 localization in the chromosome

C. jejuni prophages homologous to CJIE4 were detected in several isolates in previous work [8]. Full prophage genomes, lacking only part of the genes on either end of the prophage and the adjacent chromosomal insertion sites, were obtained from C. jejuni isolates 00-2425, 00-0949, 00-1597, 00-6200, and 01-1512 by sequencing short (2.5 to 3 kb) regions using PCR primers developed using the DNA sequence of CJIE4 from strain RM1221. These sequences were assembled, annotated, compared, and the resulting DNA and protein sequence data used to find homologous sequences from genomes deposited in sequence databases. Very recently completed draft whole genome sequences
of the five isolates were used to unambiguously determine the locations of the prophages in the respective genomes and confirm earlier sequence data (unpublished data).

For isolates 00-0949, 00-1597, 00-2425, and 00-6200, the CJIE4 prophage was located between tRNA-Met and tRNA-Phe as with all of the other CJIE4 prophages (isolate 00-2425, accession number CP006729; for the other isolates, unpublished data). However, in isolate 01-1512 CJIE4 was inserted between Cj0393c (CJE0394) and Cj0341c (CJE0396). To ensure that this was not an artifact caused by mis-assembly of the draft genome, PCR primers were developed for both the upstream and downstream regions of CJIE4, with one primer located within the last 17 nucleotides of tRNA-Met 01-1512 were detected upstream as it was previously in strain RM1221 [1]. Only the last 17 nucleotides of tRNA-Met 01-1512 were detected upstream of the CJIE1418 gene, indicating that the translocation of the prophage or some subsequent event disrupted this tRNA locus.

**CJIE4 exhibits the heterogeneity common to bacteriophages**

Searches of the NCBI Whole-genome shotgun contigs (wgs) database with the entire nucleotide sequence available for CJIE4 from 00-0949 and 00-2425 revealed two homologous prophages with extensive regions of identity to the five prophages sequenced in the current study. CJIE4 prophages were detected in additional strains by performing BLAST searches in GenBank using a number of protein sequences from the five strains sequenced in this work. In total, 12 of these additional CJIE4 sequences were used for further comparisons.

The five prophages newly sequenced for this study showed extensive regions of identity among themselves as well as with CJIE4 from strains RM1221 and S3 (Figures 1 and 2), which appeared to be prototypical phages encompassing much of the variability encoded within this temperate bacteriophage family. There were two major groups of prophage defined by an indel encoding homologs of four RM1221 genes between CJIE1438 and 1443 in one group of isolates (Group 1 sequences; Table 1, Figure 1) and five genes designated, for the purposes of this discussion, ORF6 - 10 in the second group of organisms (Group 2 sequences; Table 1, Figure 2). This latter indel had 100% nucleotide identity over 4097/4097 nt with a sequence in C. jejuni S3, which was therefore considered the prototype prophage for this group. Genes within each of the two indels in both Group 1 and Group 2 prophage sequences were transcribed in the direction opposite to the remaining prophage genome. A C. coli strain, 2348, exhibited a 2092 nt region of complete identity and a second region with partial (87%; 410/473 nt) identity within the 4097 nt indel (Figure 2). There were lower levels of identity with the upstream insertion site for CJIE1 30% identity over 496/619 nt) containing a partial sequence of panH, as well as with the KAP-family protein also present at the left end of CJIE1 [7].

The CJIE4 homolog from strain 414 (Figure 1) appeared to represent a third member of the CJIE4 prophage family due to the much greater number of gene substitutions compared with other prophages. Though it carried CJIE1439 and the CJIE1440 locus characteristic of the RM1221 group of strains it did not have any of the remaining genes from this indel, instead bearing three additional, unrelated genes transcribed in the same direction as CJIE1439 and CJIE1440 (Figure 1).

While the two indels described above appeared to define major CJIE4-family prophage groups, there was additional extensive variability in the prophages. Three isolates (C. jejuni 00-0949, 01-1512, 414) carried insertions between CJIE1420 and 1421 homologues (Figures 1 and 2). The insertion in strain 414 was larger than that of the other two isolates and had four ORFs in addition to the putative antirepressor protein. All three prophages had in common. An apparent partial duplication of CJIE1418 was found adjacent to the full-length gene in the CJIE4 prophage from strain 84-25. Genes encoding CJIE1437, CJIE1446, CJIE1456, and CJIE1463 were not annotated in several phage genomes, and CJIE1438 was additionally not found in the 1997-14 CJIE4 prophage genome. A novel gene, designated ORF12 for discussion purposes, replaced CJIE1449 in many prophages. The predicted protein sequences of ORF12 and CJIE1449 had no significant identity with each other.

Two genes (ORFs 13 and 14) encoding proteins associated with the plasmid stability system were located between CJIE1467 and CJIE1468 in CJIE4 prophages from isolates 00-2425, 00-6200, 5194, 1997-14, and 414 (Figure 1); none were found in prophages from Group 2 prophages (Figure 2). Premature stop codons were detected in several genes (see Figure 2) and some proteins were annotated as having a different size than the consensus of the group (data not shown). Strain 414, in addition to the differences discussed above, has two genes replacing CJIE1450 and 1451 and a four gene insertion between CJIE1466 and CJIE1467. The prophage from this strain, perhaps to compensate, did not carry the gene encoding the CJIE1468 capsid-associated protein (Figure 1).

Two potential prophage repressors were identified on the basis of protein sequence homology with other proteins identified as repressors in NCBI databases. Genes for ORF6 and CJIE1440 were present in the two separate indels at the same location within the CJIE4 prophage genome. A gene encoding CJIE1440 was also present in strain 414 (Figure 1) even though the indel was different in this strain, including CJIE1439 and three additional genes not found in the other CJIE4 DNA sequences included in this study. All CJIE1440 and all ORF 6 protein sequences were identical. Neither of the two putative repressor proteins exhibited detectable protein or DNA sequence identity with each other and these two proteins appeared very different to each other based on physicochemical properties inferred from their DNA sequences (Table 2). Both appeared to carry domains in common with the lambda CI repressor but not the lambda Cro repressor.

**Expression of proteins from both of the two alternate indels was detected in iTRAQ experiments**

Expression of CJIE4 prophage proteins was assessed in iTRAQ experiments with isolates grown on Mueller-Hinton agar. The intent was to determine whether any of the proteins were expressed when the prophage was integrated into the chromosome, which may therefore contribute in some way to the biology of the host bacterium in the absence of prophage induction. Proteins were detected previously in iTRAQ experiments for 00-2425 and the results have been communicated (Clark et al., submitted for publication). A second four-plex iTRAQ experiment was conducted in triplicate (three biological replicates) to assess protein expression in the remaining four C. jejuni isolates: 00-0949, 00-1597, 00-6200, and 01-1512. The detection of CJIE4-associated proteins from all five isolates, stripped of relative quantitation data and scored only on the basis of whether the proteins were identified or not, is summarized in Table 3.
The protein products of ORFs 6–10 were all detected in isolates 00-0949 and 00-1597 but not in the other three isolates. Similarly, the protein products of 3 of 4 genes in the alternate indel (CJE1339, CJE1440, and CJE1441) were detected in isolates 00-2425, 00-6200, and 00-1512. Expression of each of the putative CI-like repressors (ORF6 and CJE1440) was detected. These results were consistent with the DNA sequences obtained for these CJIE4 prophages. The expression of 12 other CJIE4 proteins could be detected in the iTRAQ experiment that included isolates 00-0949, 00-1597, 00-6200, and 01-1512, while only 8 were detected in the iTRAQ experiments that included isolate 00-2425. Among the proteins detected were: CJE1429, which contains a domain associated with transcriptional regulators; the prophage integrase and putative excisionase proteins; the major capsid protein; RloG; and emm-like protein. This suggests some level of derepression of protein expression. Three proteins homologous to CJE1444, CJE1447, and CJE1452 from strain RM1221 were extremely similar to proteins encoded by genes present in CJIE2 [1]; it was therefore not possible to determine whether these proteins were expressed from CJIE4, CJIE2, or both inserted elements. The toxin-antitoxin system protein MazF was detected in all five isolates tested, while the RelE protein was not detected in either isolate 00-2425 or isolate 00-6200. Additional hypothetical proteins with unknown function (CJE1432, CJE1466) were also detected.

Expression of proteins from other Campylobacter jejuni integrated elements (CJIEs)

Previous work has dealt extensively with the expression of CJIE1 proteins under various conditions in isolate 00-2425 (manuscript submitted for publication). Data from those experiments have been included in Table 4. Some differences in protein detection were found in different iTRAQ experiments; only data pertaining to proteins also detected in the iTRAQ experiments with isolates 00-0949, 00-1597, 00-6200, and 01-1512 were included in the table. It was apparent from the relative expression levels in iTRAQ four-plex experiments that the CJIE1 prophage, including the unique moron containing ORF11 [7], was present in isolates 00-0949 and 01-1512 (Table 4). In isolate 00-0949 most of the CJIE1 genes, including the gene encoding a homolog of the CJIE0256 dna extracellular deoxyribonuclease, were found throughout 7 of the 25 contigs in the draft genome sequence. Similarly, the gene encoding CJIE0256 and all CJIE1 genes except those encoding CJIE0262-CJIE0264 were detected in 8 of 25...
contigs comprising the draft genome of isolate 01-1512. The only CJIE2 genes detected in draft genome sequences were those that were also common with CJIE4 (CJE0590 – CJE0598 [1]), and it was clear from the context of the remaining genes in the contig that these genes belonged to CJIE4 and that CJIE2 was not present in any of the isolates. Consistent with the inability to demonstrate the presence of CJIE2, no expression of CJIE2 proteins was detected in any of the isolates used for this study.

**Discussion**

CJIE4 temperate bacteriophages showed considerable variability, and the genes associated with the largest indels were expressed. Expression of proteins associated with other genes differentially carried in CJIE4 was also detected in comparative proteomics experiments using iTRAQ labeling. Differential production of prophage proteins could potentially result in differential virulence, different biological properties of the *Campylobacter* isolates, or both. An examination of the literature suggests that many of the moron proteins associated with the CJIE4 prophage may have had roles that affected the biology of the phage itself in addition to roles in the biology of the host bacterium. Little has been published about these proteins in *C. jejuni*, so that it is necessary to attempt to infer possible effects on *C. jejuni* biology from what is known about these proteins in other bacteria.

Prophage-encoded DNases, such as the CJE1441 protein, have previously been shown to be expressed, functional, and to result in a non-naturally transformable phenotype leading to stable *Campylobacter* lineages [5,6]. Expression of the CJIE4 variant of this DNase was detected in the three isolates carrying the gene encoding it (Table 3). A second periplasmic DNase encoded by a gene within the CJIE1 prophage (dns, CJE0256) was also detected in isolates 00-0949, 00-2425, and 01-1512 (data not shown). This indicates that 00-0949 and 01-1512 carry the CJIE1 prophage in addition to isolate 00-2425, a supposition borne out by the observation that they also express four other CJIE1 proteins whereas isolates 00-1597 and 00-6200 do not. Whole genome sequencing verified these observations (unpublished data). The absence of the gene encoding the CJIE1441 DNase from isolates carrying the second CJIE4 large indel may not have detectable biological consequences in isolates with DNases encoded by prophage CJIE1 or integrated element CJIE2, which carries a DNase (CJE0566) similar to CJE1441 [1]. However, in the absence of CJIE1 and CJIE2 elements, isolates carrying CJIE4 with the indel lacking CJIE1441 may not have the ability to inhibit natural transformation and consequently may not form stable lineages [5]. It could therefore be important to know the content of prophage-encoded periplasmic endonucleases in order to interpret population studies of *Campylobacter* spp., perhaps even to assess and interpret data associated with outbreaks in human populations.

The genes encoding two distinct CJIE4-encoded putative CI-like prophage repressor proteins were present, and proteins expressed by these genes were detected in each of the five isolates.

[Figure 2. Schematic diagram demonstrating differences in Group 2 CJIE4 prophage DNA sequences.](#)

Strains carried the alternate large indel containing ORFs 6–10. The diagram includes prophages investigated in this work (see Materials and Methods) plus those obtained from NCBI databases. The question mark in the 2548 indicates that the fact that no sequence was found for this region may indicate either that there was a problem with the search parameters used to detect contigs encoding proteins homologous to those in other CJIE4 variants or that there was a horizontal gene transfer replacing the region with a different gene repertoire. The colors used to identify putative protein functions were the same as in Fig. 1. Sizes of genes are approximate, and any non-coding sequence between genes has not been shown to conserve space. doi:10.1371/journal.pone.0095349.g002
Table 1. Comparison of sequence annotation for five CJIE4 prophages.

| RM1221 | Gene/protein description | Presence (+) or absence (-) in isolate or prophage |
|--------|--------------------------|-----------------------------------------------|
| CJE1418 | intA, phage integrase, site-specific recombinase | ++ + + + + |
| CJE1419 | DNA binding protein, transcriptional regulator (excisionase) | ++ + + + + |
| CJE1420 | hypothetical protein | + + + + + |
| ORF1    | RHA family protein; antirepressor? | - - + - + |
| ORF4    | hypothetical protein | - - + - + |
| CJE1421 | dpnA, site-specific DNA methyl-transferase | + + + + + |
| CJE1422 | Emm-like protein | + + + + + |
| CJE1423 | hypothetical protein | + + + + + |
| CJE1424 | hypothetical protein | + + + + + |
| CJE1425 | hypothetical protein | + + + + + |
| CJE1426 | hypothetical protein | + + + + + |
| CJE1427 | hypothetical protein | + + + + + |
| CJE1428 | hypothetical protein | + + + + + |
| CJE1429 | hypothetical protein, transcriptional regulator domain | + + + + + |
| CJE1430 | RloG protein | + + + + + |
| CJE1431 | hypothetical protein | + + + + + |
| CJE1432 | hypothetical protein | + + + + + |
| CJE1433 | hypothetical protein | + + + + + |
| CJE1434 | YopX protein | + + + + + |
| CJE1435 | DNA binding protein Roi, antirepressor domain | + + + + + |
| CJE1436 | hypothetical protein | + + + + + |
| CJE1437 | hypothetical protein, 77 aa, opposite strand to CJE1438 | + + + + + |
| CJE1438 | hypothetical protein, 67 aa, opposite strand to CJE1437 | + + + + + |
| ORF6    | bacteriophage Cl repressor protein | - - - + + |
| ORF7    | hypothetical protein | - - - + + |
| ORF8    | Exonuclease, DNA polymerase III epsilon subunit | - - - + + |
| ORF9    | hypothetical protein | - - - + + |
| ORF10   | KAP family P-loop protein | - - - + + |
| CJE1439 | hypothetical protein | + + + - - |
| CJE1440 | signal peptidase I, phage repressor protein | + + + - - |
| CJE1441 | DNA/RNA non-specific endonuclease, nucA | + + + - - |
| CJE1442 | hypothetical protein, PLDc_N superfamily domain | + + + - - |
| CJE1443 | hypothetical protein, homolog of CJE0599 | + + + + + |
| CJE1444 | hypothetical protein, homolog of CJE0598 | + + + + + |
| CJE1445 | hypothetical protein, homolog of CJE0597 | + + + + + |
| CJE1446 | hypothetical protein, homolog of CJE0596 | + + + + + |
| CJE1447 | hypothetical protein, homolog of CJE0595 | + + + + + |
| CJE1448 | hypothetical protein, homolog of CJE0229 and CJE0594 | + + + + + |
| CJE1449 | hypothetical protein, homolog of CJE0593 | + + + - - |
| ORF12   | hypothetical protein | - - - + + |
| CJE1450 | hypothetical protein, homolog of CJE0230 and CJE0592 | + + + + + |
| CJE1451 | hypothetical protein, homolog of CJE0591 | + + + + + |
| CJE1452 | hypothetical protein, homolog of CJE0590 | + + + + + |
| CJE1453 | hypothetical protein | + + + + + |
| CJE1454 | phage head-tail joining protein | + + + + + |
| CJE1455 | hypothetical protein | + + + + + |
| CJE1456 | hypothetical protein, 90 aa, opposite strand to CJE1457 | + + + + + |
| CJE1457 | hypothetical protein, 83 aa, opposite strand to CJE1456 | + + + + + |
tested, suggesting either redundancy of function or fine control of maintenance of lysogeny in these isolates. In bacteriophage λ, the Cro protein is required for entry into the lytic cycle. None of the proteins identified in the five CJIE4 prophages characterized here had a Cro protein domain. The detection of the major capsid protein indicated that some CJIE4 prophages may be partially or wholly derepressed, though it is not clear whether or not they entered the lytic cycle and produced infectious phage particles. A protein with a function similar to Cro may be expressed, or there may be leaky expression of the capsid protein in the absence of prophage induction. Alternatively, CJIE4-family prophages may regulate switching between lysogeny and the lytic cycle differently than lambda phage.

A toxin-antitoxin protein (CJE1470) was among the CJIE4 prophage-encoded proteins detected. It was highly conserved among all strains investigated except strain 414 and contained PemK and MazF domains associated with type II toxin-antitoxin systems. Type II toxin-antitoxin systems have two genes, a toxin and an antitoxin, forming an operon; both genes are constitutively transcribed [19]. A gene encoding the second protein of this system, MazE, was not detected in the CJIE4 prophage. By cleaving specific mRNA sequences at ACA sites through a ribosome-independent mechanism when activated, MazF systems selectively inhibit the synthesis of a subset of ACA-containing proteins. This endoribonuclease has been proposed to regulate bacterial cell growth in response to stress [19,20], but is also a prokaryotic gene product that can inhibit translation in bacteria, leading to cell death when activated [19]. The functions of these systems non-selectively inhibit protein synthesis when activated [19]. The functions of C. jejuni toxin-antitoxin systems may have similar roles or functions in other bacteria, as some systems are found in both Gram-negative and Gram-positive bacteria. In C. jejuni, the MazF system has been shown to be involved in biofilm formation [23]. Further experiments will be necessary to determine whether the CJIE4-encoded toxin-antitoxin proteins may have similar roles or functions in C. jejuni.

KAP family P-loop proteins include many proteins that confer immunity to bacteriophages [24]. In prokaryotes the genes encoding these proteins appear to have been spread by plasmids, though one is present on a filamentous phage in Vibrio. They also appear to have been frequently subjected to recent pseudogene formation, which, in turn, may be associated with phages driving resistance to KAP-mediated pathways [23]. The ORF10 KAP family P-loop protein encoded by CJIE4 was homologous to the CJIE1 KAP family P-loop protein encoded by C. jejuni. Its amino acids, 39% amino acid identity, 55% conserved amino acids, and 12% gaps over 591 amino acids with an e-value

| RM1221 | Presence (+) or absence (-) in isolate or prophage |
|--------|-----------------------------------------------|
| homolog | 00-2425 | 00-6200 | 01-1512 | 00-1597 | 00-0949 |
| CJE1458 | HK 97 family major capsid protein | + | + | + | + |
| CJE1459 | HK97 phage preheader protease | + | + | + | + |
| CJE1460 | hypothetical protein | + | + | + | + |
| CJE1461 | tail tape-measure protein | + | + | + | + |
| CJE1462 | hypothetical protein | + | + | + | + |
| CJE1463 | hypothetical protein | + | + | + | + |
| CJE1464 | hypothetical protein | + | + | + | + |
| CJE1465 | DNA repair protein rad2 | + | + | + | + |
| CJE1466 | hypothetical protein | + | + | + | + |
| ORF13 | hypothetical protein | + | + | - | - |
| ORF14 | addiction module toxin, RelE/StbE family | + | + | - | - |
| CJE1467 | hypothetical protein | + | + | + | + |
| CJE1468 | HK 97 phage protein | + | + | + | + |
| CJE1469 | HK 97 portal protein | + | + | + | + |
| CJE1470 | toxin-antitoxin protein, toxin MazF | + | + | + | + |
| CJE1471 | phage terminase, large subunit | + | + | + | + |
| CJE1472 | phage terminase, small subunit | + | + | + | + |
| CJE1473 | phage HNH endonuclease | + | + | + | + |
| CJE1474 | hypothetical protein | + | + | + | + |

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of $2e^{-105}$. On the basis of the literature cited, we hypothesize that the presence of related KAP family P-loop NTPases with different primary amino acid sequences on two different prophages suggests these proteins may have evolved to confer superinfection immunity to specific prophages. An alternate possibility is that they may function to make bacteria immune to infection by lytic bacteriophages. The role(s) of these proteins in the biology of both temperate bacteriophage and the host bacteria should provide a fruitful avenue of further research.

The DNA polymerase III epsilon subunit of *E. coli* has proofreading activity essential for high fidelity DNA synthesis mediated by DNA polymerase III [25]. Silencing of dnaQ gene expression reduced the growth rate of *E. coli*, indicating that the product, DNA polymerase III epsilon subunit, affected the processivity (rate of DNA synthesis) of DNA polymerase III [26]. Overexpression of the epsilon subunit may facilitate its interaction with DNA polymerases capable of replicating imperfect DNA templates [27]. This subunit is also implicated in the induction of the *E. coli* SOS response to nalidixic acid [28]. *C. jejuni* strain RM1221 has a copy of the DNA polymerase III epsilon subunit in its chromosome (CJE0502), though this 233 amino acid protein was quite dissimilar from the ORF8 characterized in this study and present in other *C. jejuni* isolates. Since many *C. jejuni* are quite syntenic, it is quite possible that the isolates examined in this study also have a chromosomally encoded epsilon subunit and that the prophage encoded protein is a duplicate; whole genome sequencing studies of the isolates used for this work are underway and should provide an unambiguous answer. BLASTp alignments of ORF8 and CJE0502 performed using the tools in NCBI returned an Expect value of 3e-19 with only 38/94 identities and 60/94 positives, with two gaps. Both ORF8 and CJE0502 had even lower identity with the *E. coli* K12 DNA polymerase III epsilon subunit.

| Protein          | Length (a.a.) | M.W. (Da)  | Charge | Predicted pl | Functional Domains                        |
|------------------|---------------|------------|--------|--------------|-------------------------------------------|
| lambda Cro       | 67            | 7,363.46   | +5.07  | 9.78         | Cro superfamily                           |
| CJE1440          | 220           | 25,472.02  | +1.30  | 7.74         | Peptidase_S24_S26 superfamily             |
| ORF6             | 127           | 14,777.86  | -0.08  | 6.76         | Phage_CI_repr                             |
| lambda CI        | 237           | 26,211.92  | -5.98  | 4.99         | Peptidase_S24_S26 superfamily; HTH_XRE superfamily |

Table 3. Detection of proteins associated with the CJIE4 prophage after growth on Mueller-Hinton agar in iTRAQ labelling experiments.

| Identified Protein                  | Accession Number | 00-2425   | 00-6200 | 00-1512 | 00-1597 | 00-0949 |
|-------------------------------------|------------------|-----------|---------|---------|---------|---------|
| site-specific recombinase, phage integrase family CJE1418 | gi|384443628 | + | + | + | + |
| hypothetical protein CJE1420, putative excisionase | gi|384443630 | - | + | + | + |
| emm-like protein CJE1422            | gi|384443632 | - | + | + | ± |
| hypothetical protein CJE1429        | gi|57238165, gi|384443638 | + | + | + | + |
| flO6 protein, putative CJE1430       | gi|384443639 | + | + | + | + |
| hypothetical protein CJE1432        | gi|57238168, gi|384443641 | + | + | + | + |
| hypothetical protein, phage repressor protein ORF6 | gi|384443648 | - | - | - | + |
| hypothetical protein ORF7            | gi|384443649 | - | - | - | + |
| exonuclease, DNA polymerase III epsilon subunit ORF8 | gi|384443650 | - | - | - | + |
| hypothetical protein ORF9            | gi|384443651 | - | - | - | + |
| KAP family P-loop domain protein ORF10 | gi|384443652 | - | - | - | + |
| hypothetical protein CJE1439        | gi|57238175 | + | + | + | - |
| signal peptidase I, phage repressor protein CJE1440 | gi|57238176 | + | + | + | - |
| DNA/RNA non-specific endonuclease CJE1441 | gi|57238272 | + | + | + | - |
| hypothetical protein CJE1444        | gi|384443653 | - | - | + | - |
| hypothetical protein CJE1447        | gi|384443655 | - | + | + | + |
| hypothetical protein CJE1452        | gi|57238311, gi|384443660 | + | + | + | + |
| major capsid protein, HK97 family CJE1458 | gi|384443665 | + | + | ± | + |
| DNA repair protein rad2, CJE1465    | gi|384443671 | - | + | + | + |
| hypothetical protein CJE1466        | gi|57238325, gi|384443672 | + | + | + | + |
| toxin-antitoxin protein MazF, CJE1470 | gi|57238328, gi|384443676 | + | + | + | + |

Expression of proteins from *C. jejuni* isolate 00-2425 was assessed in previous work [10]; -, not detected.
abnormal tetranucleotide repeats in
[31]. It was, for instance, the only CJIE4 prophage that lacked the
strain was somewhat different than other CJIE4 prophages as well
C. jejuni
most other
jejuni
functions, but their expression was detected. How these genes or
proteins may affect either the CJIE4 prophage or the bacterial host
is unknown.
While most of the protein annotations were derived from the
use of bioinformatics tools, some proteins were annotated on the
basis of other characteristic properties widely shared among
temperate bacteriophages. The homologs of CJIE1461 were
annotated as tail tape measure proteins based on their length,
high content of 14 alpha-helical coiled-coil regions, and position in
the phage genome relative to the gene encoding the major capsid
protein [29,30]. The phage integrase associated with insertion of
phage DNA into the bacterial chromosome is usually linked to an
excisionase associated with recovery of the prophage DNA from
the bacterial chromosome; the CJE1419 homolog has therefore
been annotated as an excisionase.
Comparison of whole genome sequences determined that
C. jejuni strain 414 was somewhat phylogenetically divergent from
most other C. jejuni isolates [11]. The CJIE4 prophage from this
strain was somewhat different than other CJIE4 prophages as well
[31]. It was, for instance, the only CJIE4 prophage that lacked the
gene encoding MaxF. An examination of the distribution of
abnormal tetranucleotide repeats in Campylobacter phage genes has
suggested that some prophages may have transferred into their
hosts a long time ago and subsequently lost the ability to be
transferred, while others may still have the capacity for transfer to
different hosts [31]. Phage genes within strain 414 had 0% abnormal
tetranucleotide repeats [31], strongly suggesting that
CJIE4 had undergone an ancient integration event and subse-
quently became adapted to its host. Whether this is true for other
CJIE4 prophages as well will require further investigation.
In summary, the DNA sequence and protein expression data
together suggest that moron proteins encoded by genes within the
CJIE4 prophage could potentially alter the homeostasis of infected
bacteria. This is consistent with previously published data
indicating that at least some C. jejuni are characterized by high
genetic microdiversity resulting from recombination differences in
prophage content [32]. It further supports the hypothesis that C.
jejuni are “generalists” that can exhibit phenotypic diversity in, for
instance, their interaction with eukaryotic cells regardless of
isolation source and in the absence of large differences in gene
content [32]. The differential carriage and expression of moron
genomes within prophages may be a key component in this diversity,
though the mechanisms by which these proteins act requires
further elucidation.

Table 4. Detection of proteins associated with the CJIE1 prophage after growth on Mueller-Hinton agar in iTRAQ labelling
experiments.

| Identified Protein Accession Number | 00-2425 | 00-6200 | 00-1512 | 00-1597 | 00-0949 |
|------------------------------------|---------|---------|---------|---------|---------|
| ORF1 hotspot protein               | gi|544061942| +       | -       | +       | +       |
| phage repressor protein CJIE0215   | gi|57237226, gi|157414969, gi|384443061| +       | -       | +       | -       | +       |
| hypothetical protein CJIE0216      | gi|57237227| -       | -       | +       | -       | -       | +       |
| hypothetical protein CJIE0217      | gi|57237283, gi|384443059| -       | -       | +       | -       | -       | +       |
| major tail sheath protein CJIE0227 | gi|315124253| -       | -       | +       | -       | -       | +       |
| extracellular deoxyribonuclease CJIE0256 | gi|57237266, gi|315124233| +       | -       | +       | +       | +       |
| hypothetical protein CJIE0262      | gi|384443020| -       | -       | +       | -       | -       | +       |
| transcriptional regulator CJIE0272  | gi|57237282| +       | -       | +       | +       | +       | +       |
| hypothetical protein CJIE0273      | gi|57237283| -       | -       | +       | +       | +       | +       |

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References

1. Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Radio DA, et al. (2005)
Major structural differences and novel potential virulence mechanisms from the
genomes of multiple Campylobacter species. PLoS Biol 3: e15.
2. Parker CT, Quiñones B, Miller WG, Horn ST, Mandrell RE. (2006)
Comparative genomic analysis of Campylobacter jejuni strains reveals diversity

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Author Contributions

Conceived and designed the experiments: CGC GRW. Performed the
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due to genomic elements similar to those present in C. jejuni strain RM1221. J Clin Microbiol 44: 4125–4135.

3. Barton C, Ng L-K, Tyler SD, Clark CG (2007) Temperate bacteriophages affect pulsed-field gel electrophoresis patterns of Campylobacter jejuni. J Clin Microbiol 45: 386–391.

4. Scott AE, Timms AR, Connerton PL, Carrillo CL, Radzum KA, et al. (2007) Genome dynamics of Campylobacter jejuni in response to bacteriophage predation. PLoS Pathog 3: e119.

5. Gaasbeek EJ, Wagenaar JA, Guillhabert ML, Westen MSM, van Putten JPM, et al. (2009) DNase encoded by integrated element CJIE1 inhibits natural transformation of Campylobacter jejuni. J Bacteriol 191: 2296–2306.

6. Gaasbeek EJ, Wagenaar JA, Guillhabert ML, van Putten JPM, Parker CT, et al. (2010) NudC families encoded by the integrated elements CJIE2 and CJIE4 inhibit natural transformation of Campylobacter jejuni. J Bacteriol 192: 936–941.

7. Clark CG (2011) Sequencing of CJIE1 prophages from Campylobacter jejuni reveals the presence of inserted and (oe) deleted genes. Can J Microbiol 57: 795–809.

8. Clark CG, Ng L-K (2008) Sequence variability of Campylobacter jejuni temperate bacteriophages. BMC Microbiol 8: 49.

9. Clark CG, Grant CCR, Pollari F, Marshall B, Moses J, et al. (2012) Effects of the Campylobacter jejuni CJIE1 prophage homologs on adherence and invasion in culture, patient symptoms, and source of infection. BMC Microbiol 12: 209.

10. Clark CG, Chang PM, McCoirist SJ, Simon P, Walker M, et al. 2014 The CJIE1 prophage of Campylobacter jejuni affects protein expression in growth media with and without bile salts. BMC Microbiol 14: 70.

11. Boyd EF, Carpenter MR, Chowdhury M (2012) Mobile effector proteins on genome sequence of Campylobacter jejuni 5–7. J Clin Microbiol 44: 4093–4103.

12. Hepworth PJ, Ashelford KE, Hinds J, Gould KA, Witney AA, et al. (2011) Comparative genome-wide analysis of pulsed-field gel electrophoresis patterns of Campylobacter jejuni. J Clin Microbiol 49: 1891–1898.

13. Pittenger LG, Frye JG, McNerney V, Reeves J, Haro J, et al. (2012) Analysis of Campylobacter jejuni to identify markers for different disease outcomes. J Microbiol Methods 90: 170–175.

14. Cooper KK, Cooper MA, Zuccolo A, Law B, Jorm LA (2011) Complete genome sequence of Campylobacter jejuni strain S3. J Bacteriol 193: 1491–1492.

15. Salama S, Bolton FJ, Hutchinson DN (1989) Improved method for the isolation of Campylobacter jejuni and Campylobacter coli bacteriophages. Lett Appl Microbiol 8: 5–7.

16. Clark CG, Bryden L, Cuff W, Johnson PL, Janieson F, et al. (2005) Use of the Oxford multilocus sequence typing protocol and sequencing of the flagellin short variable region to characterize isolates from a large outbreak of waterborne Campylobacter jejuni sp. strains in Walkerton, Ontario, Canada. J Clin Microbiol 43: 2080–2091.

17. Clark CG, Price L, Ahmed R, Woodward DL, Melito PL, et al. (2003) Characterization of waterborne outbreak-associated Campylobacter jejuni, Walkerton, Ontario, Emerg Infect Dis 9: 1232–1241.

18. Winiewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation for proteome analysis. Nature Methods 6: 359–362.

19. Yamaguchi Y, Park J-H, Inouye M (2011) Toxin-antitoxin systems in bacteria and Archaea. Ann Rev Genet 45: 61–79.

20. Zhang Y, Zhang J, Hara H, Kato I, Inouye M (2005) Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase. J Biol Chem 280: 3134–3130.

21. Vesper O, Amitai S, Belitsky M, Byrgazov K, Kaberdina AC, et al. (2011) Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in Escherichia coli. Cell 147: 147–157.

22. Lenherr H, Maguin E, Jalal S, Yarmolinsky MB (1993) Plasmid addiction genes of bacteriophage Pl: dv, which causes cell death on curing of prophage, and pdf, which prevents host death when prophage is retained. J Mol Biol 233: 414–420.

23. Kolodkin-Gal I, Verdi R, Schlossberg-Feldlau A, Engelberg-Kralik H (2009) Differential effect of E. coli toxin-antitoxin systems on cell death in liquid medium and biofilm formation. PLoS One 4: e6785.

24. Aravin I, Iyer NM, Leipe DD, Konnin EV (2004) A novel family of NTAPses with an unusual phylactic distribution and membrane segments inserted within the NTAPse domain. Genome Biol 5: R30.

25. McHenry CS (2011) DNA replicases from a bacterial perspective. Ann Rev Biochem 80: 403–436.

26. Stefan A, Reggiani L, Ciancetta S, Radeghieri A, Gonzalez Vara Rodriguez A, et al. (2003) Silencing of the gene coding for the epsilon subunit of DNA polymerase III slows down the growth rate of Escherichia coli populations. FEBS Lett 546: 295–299.

27. Sutton MD, Murli S, Opperman T, Klein C, Walker GC (2001) umaDC-dumQ interaction and its implications for cell-cycle regulation and SOS mutagenesis in Escherichia coli. J Bacteriol 183: 1085–1089.

28. Polhaus JR, Long DT, O'Reilly E, Kreutzer KN (2008) The t subunit of DNA polymerase III involved in the naldixid acid-induced SOS response in Escherichia coli. J Bacteriol 190: 3239–3247.

29. Veesler D, Cambillau C (2011) A common evolutionary origin for tailed bacteriophage functional modules and bacterial machineries. Microbiol Mol Biol Rev 75: 423–433.

30. Stefan A, Reggiani L, Ciancetta S, Radeghieri A, Gonzalez Vara Rodriguez A, et al. (2003) Silencing of the gene coding for the epsilon subunit of DNA polymerase III slows down the growth rate of Escherichia coli populations. FEBS Lett 546: 295–299.

31. Sutter MD, Murli S, Opperman T, Klein C, Walker GC (2001) umaDC-dumQ interaction and its implications for cell-cycle regulation and SOS mutagenesis in Escherichia coli. J Bacteriol 183: 1085–1089.

32. Polhaus JR, Long DT, O’Reilly E, Kreutzer KN (2008) The t subunit of DNA polymerase III involved in the naldixid acid-induced SOS response in Escherichia coli. J Bacteriol 190: 3239–3247.

33. Veesler D, Cambillau C (2011) A common evolutionary origin for tailed bacteriophage functional modules and bacterial machineries. Microbiol Mol Biol Rev 75: 423–433.

34. Xu J, Hendrix RW, Duda RL (2004) Conserved translational frameshift in fdDNA bacteriophage tail assembly genes. Mol Cell 16: 11–21.

35. Zhou Y, Bu L, Gao M, Zhou C, Wang Y, et al. (2013) Comprehensive genomic characterization of Campylobacter jejuni reveals some underlying mechanisms for its genomic diversification. PLoS One 8(8): e70241.

36. Gripp E, Hidalgo D, Delectot X, Kops F, Maurinchat S, et al. (2011) Closely related Campylobacter jejuni strains from different sources reveal a generalist rather than a specialist lifestyle. BMC Genom 12: 584.