



Biofilm Formation and Motility Are Promoted by Cj0588-Directed Methylation of rRNA in *Campylobacter jejuni*

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Numerous bacterial pathogens express an ortholog of the enzyme TlyA, which is an rRNA 2'-O-methyltransferase associated with resistance to cyclic peptide antibiotics such as capreomycin. Several other virulence traits have also been attributed to TlyA, and these appear to be unrelated to its methyltransferase activity. The bacterial pathogen *Campylobacter jejuni* possesses the TlyA homolog Cj0588, which has been shown to contribute to virulence. Here, we investigate the mechanism of Cj0588 action and demonstrate that it is a type I homolog of TlyA that 2'-O-methylates 23S rRNA nucleotide C1920. This same specific function is retained by Cj0588 both *in vitro* and also when expressed in *Escherichia coli*. Deletion of the *cj0588* gene in *C. jejuni* or substitution with alanine of K⁸⁰, D¹⁶², or K¹⁸⁸ in the catalytic center of the enzyme cause complete loss of 2'-O-methylation activity. Cofactor interactions remain unchanged and binding affinity to the ribosomal substrate is only slightly reduced, indicating that the inactivated proteins are folded correctly. The substitution mutations thus dissociate the 2'-O-methylation function of Cj0588/TlyA from any other putative roles that the protein might play. *C. jejuni* strains expressing catalytically inactive versions of Cj0588 have the same phenotype as *cj0588*-null mutants, and show altered tolerance to capreomycin due to perturbed ribosomal subunit association, reduced motility and impaired ability to form biofilms. These functions are reestablished when methyltransferase activity is restored and we conclude that the contribution of Cj0588 to virulence in *C. jejuni* is a consequence of the enzyme's ability to methylate its rRNA.

Keywords: TlyA 2'-O-methyltransferase, biofilms, bacterial motility, virulence, capreomycin resistance

INTRODUCTION

Proteins encoded by orthologs of *tlyA* genes are present in a diverse range of bacterial pathogens including *Mycobacterium* sp., *Campylobacter jejuni* and *Brachyspira (Serpulina) hyodysenteriae*, and have been linked to various roles in virulence including hemolysis (Wren et al., 1998) and antibiotic resistance (Maus et al., 2005). The role of TlyA in resistance to the clinically important

tuberactinomycin antibiotics capreomycin and viomycin is associated with its function as an rRNA methyltransferase (Johansen et al., 2006). TlyA 2'-O-methylates specific cytidines within the ribosomal binding site of capreomycin and viomycin. Inactivation of TlyA, with consequent loss of methylation, confers resistance in bacterial pathogens including *Mycobacterium tuberculosis* (Maus et al., 2005; Johansen et al., 2006; Monshupanee et al., 2012). Less clear, however, is how TlyA might engage in other aspects of virulence such as hemolysis that have been ascribed to this protein (Wren et al., 1998; Monshupanee, 2013).

Alignments of TlyA sequences from various organisms show that they possess a conserved K-D-K-E amino acid tetrad previously identified at the active core of other 2'-O-methyltransferases such as RrmJ/FtsJ (Bugl et al., 2000; Hager et al., 2002; Feder et al., 2003; Punekar et al., 2012). These structural features are also evident in the TlyA ortholog Cj0588 of *C. jejuni*. We show here that Cj0588 belongs to the type I group of TlyA (TlyA^I) enzymes, which 2'-O-methylate solely at 23S rRNA nucleotide C1920 as previously seen for the *B. hyodysenteriae* and *Thermus thermophilus* homologs (Monshupanee et al., 2012). Slightly longer TlyA^{II} versions with sequence extensions at their N- and C-termini are found in actinobacterial species including *M. tuberculosis*, and these enzymes 2'-O-methylate not only nucleotide C1920 but also nucleotide C1409 in 16S rRNA (Johansen et al., 2006; Monshupanee et al., 2012). Nucleotides C1409 and C1920 are respectively located on the interface of the 30 and 50S ribosomal subunits and come into close proximity at the capreomycin/viomycin binding site upon subunit association (Johansen et al., 2006; Stanley et al., 2010).

In this study, we engineered Cj0588/TlyA in pathogenic strains of *C. jejuni* to produce experimental model systems for assessing the role of the protein in physiological traits such as motility and biofilm formation that have been linked with virulence (Young et al., 2007; Bolton, 2015). Biofilms are resilient structures that account for the prevalence of *C. jejuni* in food processing environments (Reuter et al., 2010), and are directly involved in human disease by facilitating adhesion of *C. jejuni* and its colonization of the mucus lining of the intestinal tract (Haddock et al., 2010). Adherence of *C. jejuni* to human colorectal epithelial cells has previously been shown to be impaired after loss of *cj0588* (Salamaszyńska-Guz and Klimuszko, 2008), although it remained unclear how *cj0588* might function in this manner.

The motility of *C. jejuni* and its ability to form biofilms in the environment and during host infection are processes that can be reproduced and studied under laboratory conditions. Many commonly known *C. jejuni* strains that are pathogenic in humans (Epps et al., 2013) have been isolated from cases of zoonotic infection acquired from poultry and other animals (Luethy et al., 2017). *C. jejuni* 405 and 81-176 (Turonova et al., 2015) are representative strains with the former being the more motile and the latter more proficient at forming biofilms. We have engineered both strains with a series of amino acid substitution within the active site of the Cj0588 enzyme to cause loss of its TlyA^I methyltransferase activity without affecting the overall structure of the protein. Methylation of the *C. jejuni* rRNA

was followed by biochemical and mass spectrometric approaches and was correlated with changes in phenotypic traits including motility and biofilm morphology.

MATERIALS AND METHODS

Modeling of the Cj0588/TlyA Methyltransferase Structure

The *C. jejuni* TlyA homolog Cj0588 (A0A0M4TPK5) was identified in BLAST searches (Altschul et al., 1997) using the *Mycobacterium tuberculosis* (P9WJ63) and *Brachyspira* (formerly *Serpulina*) *hyodysenteriae* (Q06803) TlyA sequences as queries (Monshupanee et al., 2012). Sequences were aligned with Clustal Omega (Sievers et al., 2011). Modeling of the tertiary structure of the Cj0588 catalytic domain was carried out with Modeller version 9.19 (Sali and Blundell, 1993) in UCSF Chimera (Pettersen et al., 2004) using *M. tuberculosis* TlyA (PDB: 5EOV) (Witek et al., 2017) as a reference to fold the Cj0588 peptide chain. The HhaI methyltransferase structure (PDB: 2HMY) was used to superimpose the AdoMet cofactor (O'Gara et al., 1999).

Bacterial Strains, Plasmids, Media, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *C. jejuni* strains were grown at 37°C on Mueller-Hinton (MH, bioMérieux) agar containing 5% (v/v) sheep blood under microaerobic conditions using BD GasPak EZ CO₂ sachets (Becton Dickinson). *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) broth or agar (bioMérieux) supplemented with chloramphenicol at 20 μg ml⁻¹ and/or kanamycin at 25 μg ml⁻¹.

Transformation of *C. jejuni*

Campylobacter jejuni electrocompetent cells (Wassenaar et al., 1993) were mixed with plasmid DNA (0.5–5 μg) and electroporated using an Electro Cell Manipulator (ECM 600) at 50 μF, 126 Ω and 1.25 kV. Transformants were grown under microaerobic conditions at 37°C on MH plates for 5 h and then replated and grown for a further 2–5 days on MH agar containing 5% (v/v) sheep blood supplemented with chloramphenicol at 20 μg ml⁻¹ and/or kanamycin at 25 μg ml⁻¹.

Construction and Complementation of *C. jejuni* *cj0588*-Null Strains

Null mutants of the *C. jejuni* strains 405 and 81-176 were created by inserting a chloramphenicol *cat* resistance cassette (Cm^r) into *cj0588* (*tlyA*). Briefly, the *cj0588* gene and flanking regions were amplified by PCR using the C5F and C5R primers (Table S2) and cloned into the pBluescript II SK plasmid; 450 bp of the *cj0588* coding sequence was then replaced with a *cat* cassette. The plasmids lack a *C. jejuni* replicon, and thus retention of Cm^r after electrotransformation of the *C. jejuni* strains requires homologous allelic exchange with the chromosomal *cj0588* gene. Recombinant plasmid structures were tested at each stage by restriction digestion; in all cases, plasmids and

strains (Table S1) were finally verified by PCR sequencing (Table S2).

The *C. jejuni* 405 Δ *cj0588* null-mutant was complemented *in trans* with plasmid-encoded versions of the *cj0588* gene. A fragment containing the *cj0588* gene was amplified by PCR from *C. jejuni* 81-176 using primers C2 and C5R (Table S2) and ligated into the *Xba*I-digested shuttle vector p0183 under *C. jejuni* promoter control to form plasmid pMW0588 (Salamaszyńska-Guz et al., 2013). This plasmid was used to transform *E. coli* before being moved to *C. jejuni* 405 forming strain 405 Δ *cj0588*+pMW0588 (Table S1). The *C. jejuni* 81-176 null-mutant was complemented by inserting a wild-type copy *cj0588* under *C. jejuni* promoter control into the 121-bp intergenic region between *cj0652* and *cj0653c* (Javed et al., 2012) forming the *C. jejuni* strain 81-176 Δ *cj0588::0588* (Table S1). Strains of 81-176 expressing the mutant versions of *cj0588* (described below) were created in the same way.

Mutagenesis of the Cj0588 Active Site

The *cj0588* gene was mutagenized in plasmids pET0588 and pMW0588 using PCR primers (Table S2) and the QuikChange II (Agilent) protocol to individually substitute the K⁸⁰, D¹⁶², and K¹⁸⁸ codons with alanine codons.

Minimal inhibitory concentrations (MICs) were determined by diluting overnight cultures of *C. jejuni* strains and plating on MH sheep blood agar with 2-fold steps in drug concentration. MIC values are the lowest concentration at which no growth was observed after incubation under microaerobic conditions for 48 h at 37°C.

Polysome profiles of the *C. jejuni* Cj0588 recombinants were determined on sucrose gradients (Douthwaite et al., 1989) under nonstringent salt conditions [20 mM Tris-HCl pH 7.5, 60 mM NH₄Cl, 10.5 mM Mg(CH₃COO)₂, 0.1 mM EDTA, 2 mM β -mercaptoethanol].

Methylation of rRNA *in Vivo*

Ribosomal subunits were prepared from *C. jejuni* strains on sucrose gradients for isolation of rRNAs, as described previously for *E. coli* (Douthwaite et al., 1989). 5'-³²P-end-labeled deoxynucleotide primers were hybridized to complementary regions of 16S rRNA nucleotides 1411–1429 and 23S rRNA nucleotides 1926–1943, extended with AMV reverse transcriptase (Roche) and run on polyacrylamide/urea gels to detect sites of 2'-*O*-methylation (Maden et al., 1995; Johansen et al., 2006).

In MALDI-ToF (matrix assisted laser desorption/ionization time-of-flight) mass spectrometry analyses, the oligodeoxynucleotides SR199 and SJ20 (Table S2) were used to assess methylation in the *C. jejuni* rRNAs (Andersen et al., 2004; Douthwaite and Kirpekar, 2007). MALDI-ToF mass spectrometry data for rRNA samples fragmented with RNase A or T1 were collected in positive mode (UltrafleXtreme, Bruker).

Analysis of *in Vitro* Methylation of rRNA

Histidine-tagged versions of the Cj0588 methyltransferase (Table S1) were purified from recombinant *E. coli* BL21(DE3) cells (Salamaszyńska-Guz and Klimuszek, 2008). Methylation

of rRNA by 250–500 nM purified Cj0588 protein was assayed in 50 mM Tris-Cl, pH 7.5, 10 mM EDTA, 10 mM β -mercaptoethanol, and 25 mM NaCl. The K_m for *S*-adenosyl-L-methionine (AdoMet) was determined using 6 μ M 50S ribosomal subunits as substrate and increasing amounts of [³H]-AdoMet (10.0 Ci mmol⁻¹ = 3.7 \times 10¹¹ Bq mmol⁻¹; Amersham Biosciences). The K_m for the substrate was determined varying the concentration of 50S ribosomal subunits in the presence of 10 μ M AdoMet for 30 min at 37°C. The components were then spotted onto DE81 filter paper discs (Whatman, Brentford, UK) followed by air-drying and washing three times for 10 min with 50 mM KH₂PO₄, once with water, and finally with 70% ethanol. Incorporation of ³H-labeled methyl was measured by liquid scintillation (Wallace, Pharmacia), and for kinetic analysis Origin software was used (OriginLab).

Motility and Biofilm Assays

The motility of *C. jejuni* cells was followed by adding 3 μ l of culture (OD₆₀₀ 0.5) onto MH medium with 0.25% agar. Plates were left to dry and were incubated under microaerobic conditions for 48 h at 37°C before measuring cell migration.

Biofilm formation by the *C. jejuni* 405 and 81-176 strains was first assessed by staining. Strains were grown overnight in Mueller-Hinton broth at 37°C under microaerobic conditions in 24-well polystyrene plates. The broth was removed and cells were stained with 0.1% crystal violet for 5 min at room temperature. Unbound stain was removed by washing with water, and samples were dried and decolorized with ethanol/acetone before measuring absorbance at 570 nm using a BioMate spectrophotometer (Thermo Scientific).

Independent visualization of biofilms was carried out by Field Emission Scanning Electron Microscopy (FESEM). *C. jejuni* 81-176 recombinants were grown on Columbia agar plates for 24 h before harvesting and suspending in 5 ml BHI broth (OD₆₀₀ = 0.05) and cultivating for 48 h at 37°C in 5% CO₂ on glass cover slides. Biofilms were fixed for 24 h in 0.1 M cacodylate buffer (pH 7.3) with 3% glutaraldehyde followed by washing for 60 min in cacodylate buffer without glutaraldehyde, and then four times for 30 min in fresh buffer followed by dehydration for 6 h in 96% ethanol. Biofilms were air-dried and coated with gold-palladium (2–4 nm thick) and analyzed at nanometer image resolution by FESEM (MERLIN Carl Zeiss Germany) at 2–5 kV range accelerating voltage.

RESULTS

The *C. jejuni* Cj0588 Protein Is a TlyA^I Methyltransferase

The sequence of the Cj0588 protein indicates that it belongs to the TlyA^I group, the members of which have a single methylation target on the 2'-*O*-position of 23S rRNA nucleotide C1920. In comparison with the orthologous group of TlyA^{II}-type enzymes that methylate not only C1920 but also nucleotide C1409 in 16S rRNA (Monshupanee et al., 2012), Cj0588 lacks four amino acids (A2-R3-R4-A5) at its N-terminus and about twenty amino acids at its C-terminus (Figure 1A). Three-dimensional modeling of the catalytic domain of Cj0588 (Figure 1B) reveals a structure

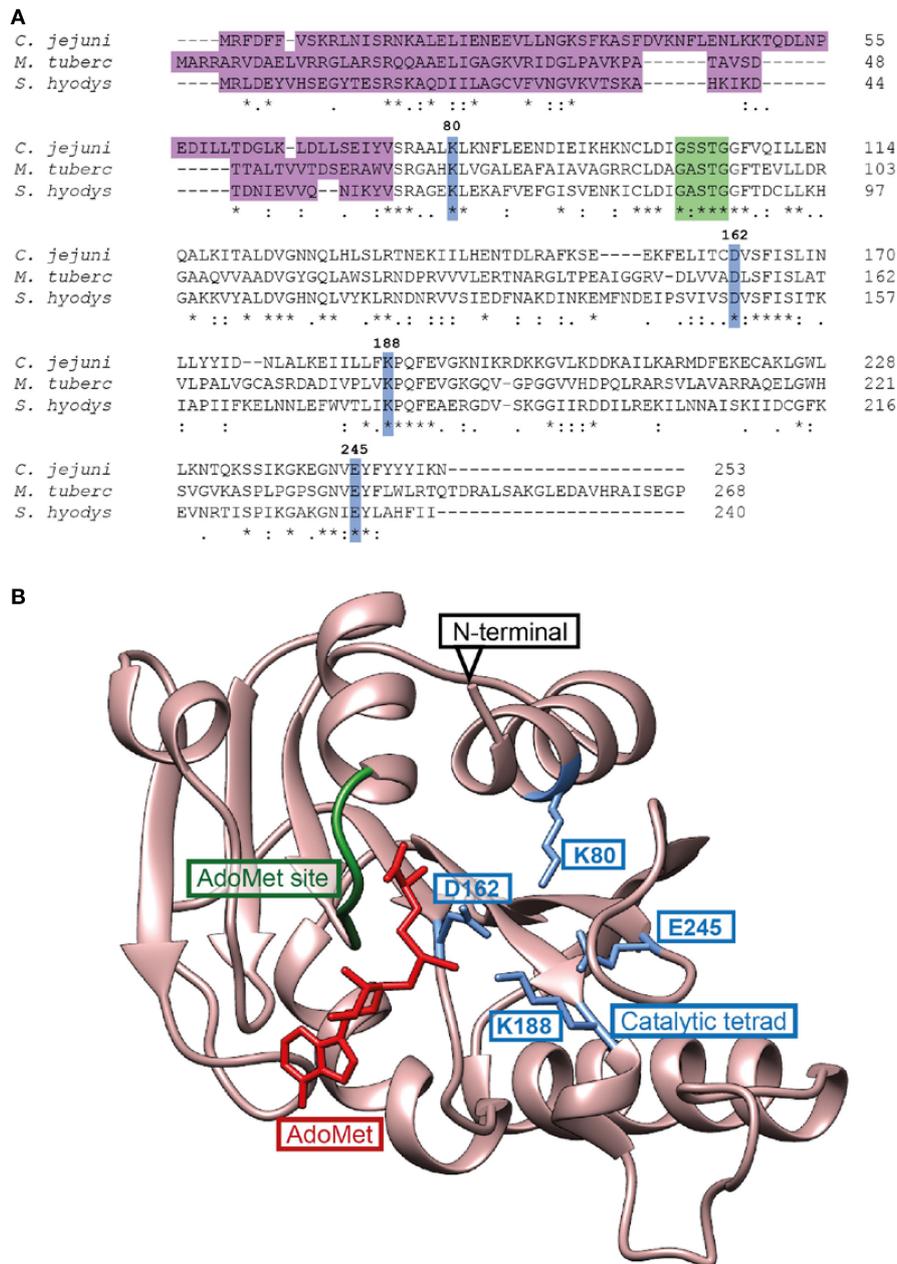


FIGURE 1 | Structure of the *C. jejuni* TlyA methyltransferase homolog Cj0588. **(A)** Multiple alignment of the *C. jejuni* TlyA homolog Cj0588 (A0A0M4TPK5) with the *M. tuberculosis* type II TlyA (P9WJ63) and the *S. hyodysenteriae* (Q06803) type I TlyA sequences. The four extra residues at the N-terminus and the C-terminal extension are hallmarks of TlyA^{II} enzymes that modify 16S rRNA nucleotide C1409 in addition to 23S rRNA C1920 (Monshupanee et al., 2012). The S4 RNA binding domain of the proteins is shown in magenta. Key amino acid residues within the catalytic center are highlighted in blue (Cj0588 residue numbering), and a portion of the AdoMet cofactor binding site is shown in green. Highly conserved residues are indicated with asterisks. Sequences were aligned with Clustal Omega (Sievers et al., 2011). **(B)** Model of the catalytic domain of the Cj0588 structure based on the 1.7 Å resolution crystal structure of the *M. tuberculosis* TlyA (PDB: 5EOV) (Witek et al., 2017). The N-terminal S4 RNA binding domain is missing in the structure (Witek et al., 2017). The catalytic tetrad (blue); in this study, three of these residues, K80, D162 and K188, were individually substituted with alanine. The AdoMet cofactor (red) was positioned alongside residues in the highly conserved AdoMet site (green) using the HhaI methyltransferase structure (PDB: 2HMY) (O’Gara et al., 1999).

typical for 2'-O-methyltransferases with a seven-stranded β -sheet between five α -helix layers and four residues K⁸⁰, D¹⁶², K¹⁸⁸ and E²⁴⁵ that comprise the catalytic center (Arenas et al., 2011; Salamaszyńska-Guz et al., 2014).

The predictions about the specificity of nucleotide methylation were tested by analyzing the rRNAs of *C. jejuni* 405 and 81-176 strains with and without the Cj0588 protein. MALDI-ToF mass spectrometric analysis of 16S rRNA from wild-type

strains showed that there was no methylation at nucleotide C1409 (Figure S1), whereas a clear top was seen corresponding to the fragment containing 2'-*O*-methylation at 23S rRNA nucleotide C1920 (Figure 2A). These findings were confirmed by primer extension (Figure 3). As anticipated, there was no

methylation at these nucleotides in the *cj0588*-null mutants, and methylation capacity was restored by complementation of null-mutants with an intact copy of the *C. jejuni* *cj0588* gene (Figure 3). The rRNA methylation analyses together with the *in silico* studies of the *C. jejuni* Cj0588 structure

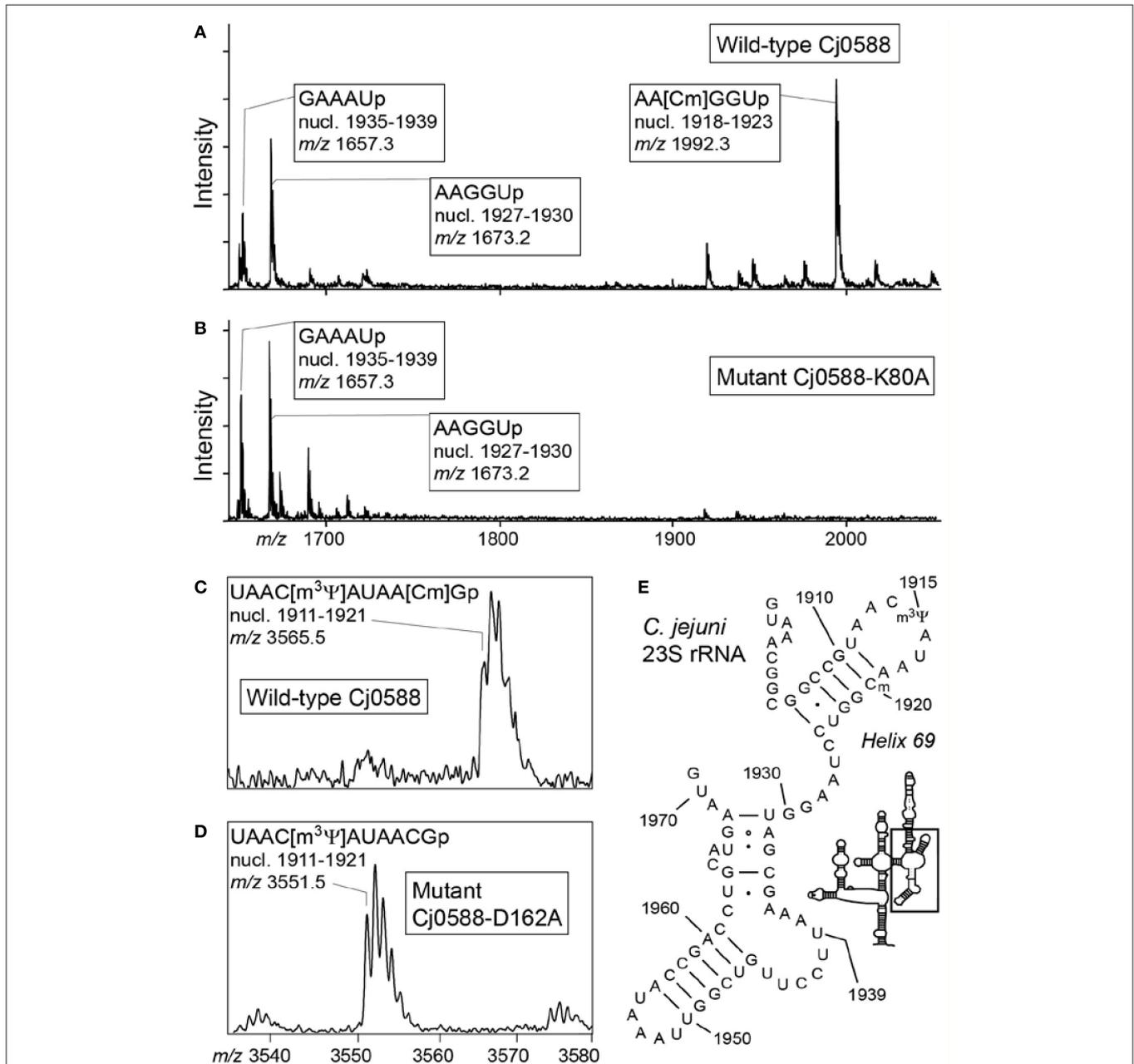
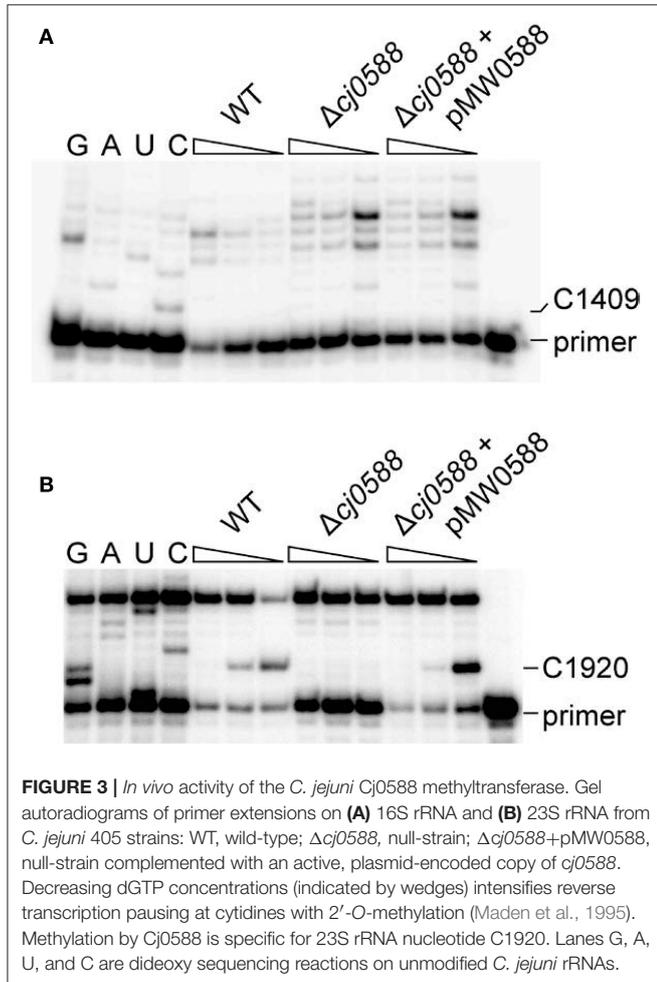


FIGURE 2 | Mass spectrometric analyses of *C. jejuni* 23S rRNA. **(A)** Spectrum of RNase A fragments derived from the 50-nucleotide sequence encompassing C1920 in the 23S rRNA from the *C. jejuni* 405 wild-type strain. Nucleotide C1920 is evident in the AA[Cm]GGUp fragment at mass/charge (*m/z*) 1992, where 2'-*O*-methylation prevents RNase A cleavage. **(B)** This hexamer sequence is unmethylated in rRNA from the Cj0588-K80A mutant and is consequently cleaved to smaller AACp and GGUp fragments. The D162A and K188A strains produced fragmentation patterns identical to K80A (not shown). **(C)** After RNase T1 digestion, Cm1920 appears in the 11-mer fragment of nucleotides 1911–1921 together with nucleotide $m^3\psi$ 1915. **(D)** The corresponding fragment from the Cj0588-D162A mutant showed that a single methyl group was missing. The K80A and K188A mutants (not shown) produced spectra identical to D162A. **(E)** Helix 69 and surrounding structures in the 23S rRNA (boxed in the schematic) including the sequence that was analyzed by MS. The m^3U 1939 modification, which is present in most bacteria (Desmolaize et al., 2011), is missing in *C. jejuni* (the unmodified nucleotide is evident in the GAAAUp fragment in **A,B**).

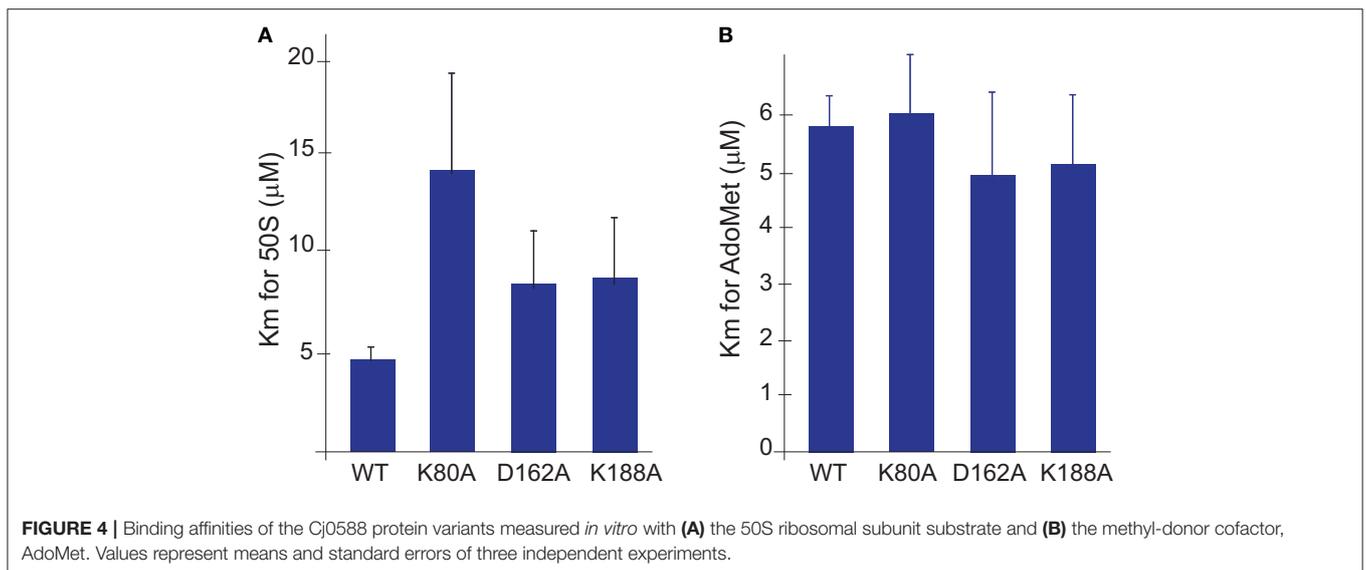
establish that this protein belongs to the TlyA^I group of RNA methyltransferases that modify solely at 23S rRNA nucleotide C1920.

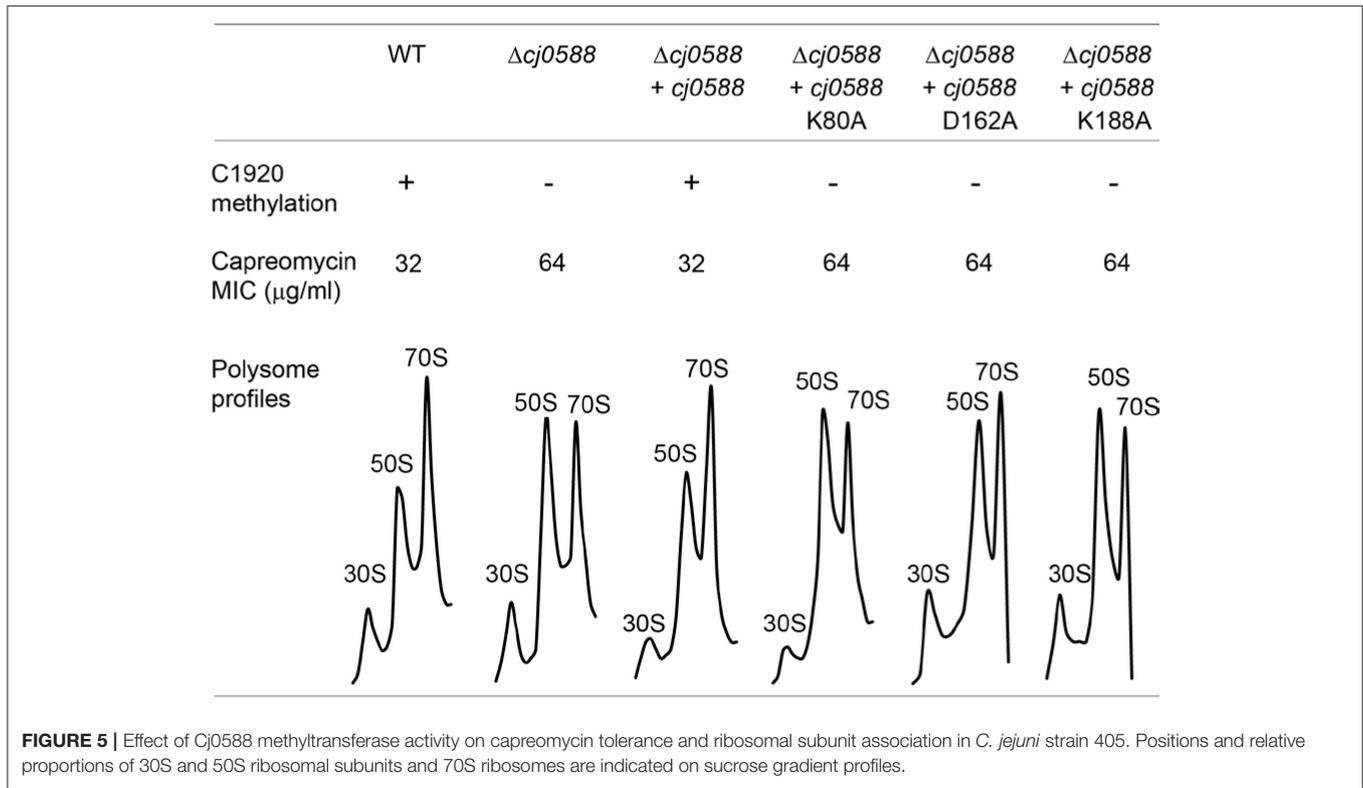


Additional Modifications within Antibiotic Binding Regions of *C. jejuni* rRNAs

The mass spectrometry data revealed several additional modifications within helix 44 of *C. jejuni* 16S rRNA and helix 69 of 23S rRNA, where interactions with diverse groups of antibiotics occur (Johansen et al., 2006; Wilson, 2009). In 16S rRNA, two methyl groups on nucleotide C1402 and an additional methylation at C1404 were evident (Figures S1A,B), and there were no further modifications at other nucleotides in this sequence. These 16S rRNA methylations are presumably added by enzymes encoded by the *C. jejuni* genes *cj0636*, *cj0693c*, and *cj0154c* that are homologous to the *E. coli* rRNA methyltransferase genes *rsmF*, *rsmH*, and *rsmI* (Figure S1E). The *E. coli* RsmH and RsmI enzymes respectively methylate the base and ribose of C1402 (Kimura and Suzuki, 2010), and have 32 and 35% identity with the homologous *C. jejuni* proteins. The m⁵C1404 modification is presumably added by Cj0636, which is orthologous to NOL1/NOP2/sun proteins including the *Enterococcus faecium* methyltransferase (35% identical) that modifies the same 16S rRNA nucleobase (Galimand et al., 2011), and *E. coli* RsmF that methylates the 5-position of the neighboring nucleotide C1407 (Andersen and Douthwaite, 2006).

In 23S rRNA, U1915 is converted to m³ψ (Figure 2C) by the enzymes encoded by the *C. jejuni* genes *cj1280c* and *cj0126c* that are equivalent to the *E. coli* pseudouridine synthetase RluD (Huang et al., 1998; Wrzesinski et al., 2000) and methyltransferase RlmH (Ero et al., 2008; Purta et al., 2008), respectively. No modification was observed at nucleotide U1939 (Figure 2A), which is commonly methylated in many bacteria (Agarwalla et al., 2002; Madsen et al., 2003), and consistent with this, no homolog of the *rlmD* gene that encodes the m⁵U1939 methyltransferase enzyme is evident in the *C. jejuni* genome.





Substitutions in the Cj0588/TlyA Catalytic Center Eliminate Methylation Activity

Three of the four key residues in the catalytic tetrad were individually substituted with alanine to eliminate methyltransferase activity without perturbing the overall structure of the protein. Assaying the *C. jejuni* mutant Cj0588 enzymes under *in vitro* conditions where the substrate and cofactor were present in saturating amounts showed that binding affinity for the AdoMet cofactor was not significantly altered by the K80A, D162A, and K188A substitutions (Figure 4). These substitutions had a mild effect on the binding affinity for the 50S subunit substrate, increasing the K_m between 2- and 3-fold (Figure 4).

Expression of the mutant Cj0588 enzymes *in vivo* in *C. jejuni* followed by rRNA analysis by mass spectrometry and primer extension showed that each of the K80A, D162A and K188A substitutions led to complete loss of rRNA methylation (Figure 2, Figure S2). The Cj0588 proteins behaved in an identical manner when expressed in *E. coli* where the wild-type enzyme methylated effectively at 23S rRNA C1920, and with no methylation at this nucleotide in strains expressing the mutant enzymes (data not shown).

In *M. tuberculosis*, loss of ribose methylation at nucleotide C1920 and/or C1409 affects the interaction between capreomycin and the ribosome sufficiently to confer clinical resistance (Maus et al., 2005; Johansen et al., 2006). In *C. jejuni*, loss of Cj0588-directed methylation increases the MIC of capreomycin by 2-fold and interferes with the association of ribosomal subunits to form functional 70S ribosomes (Figure 5), although this was

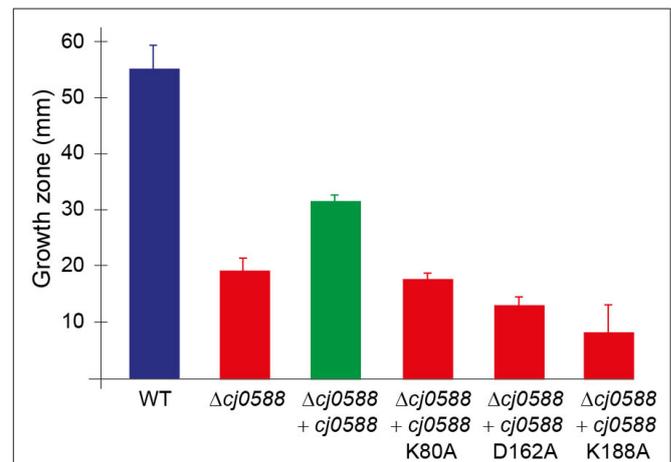
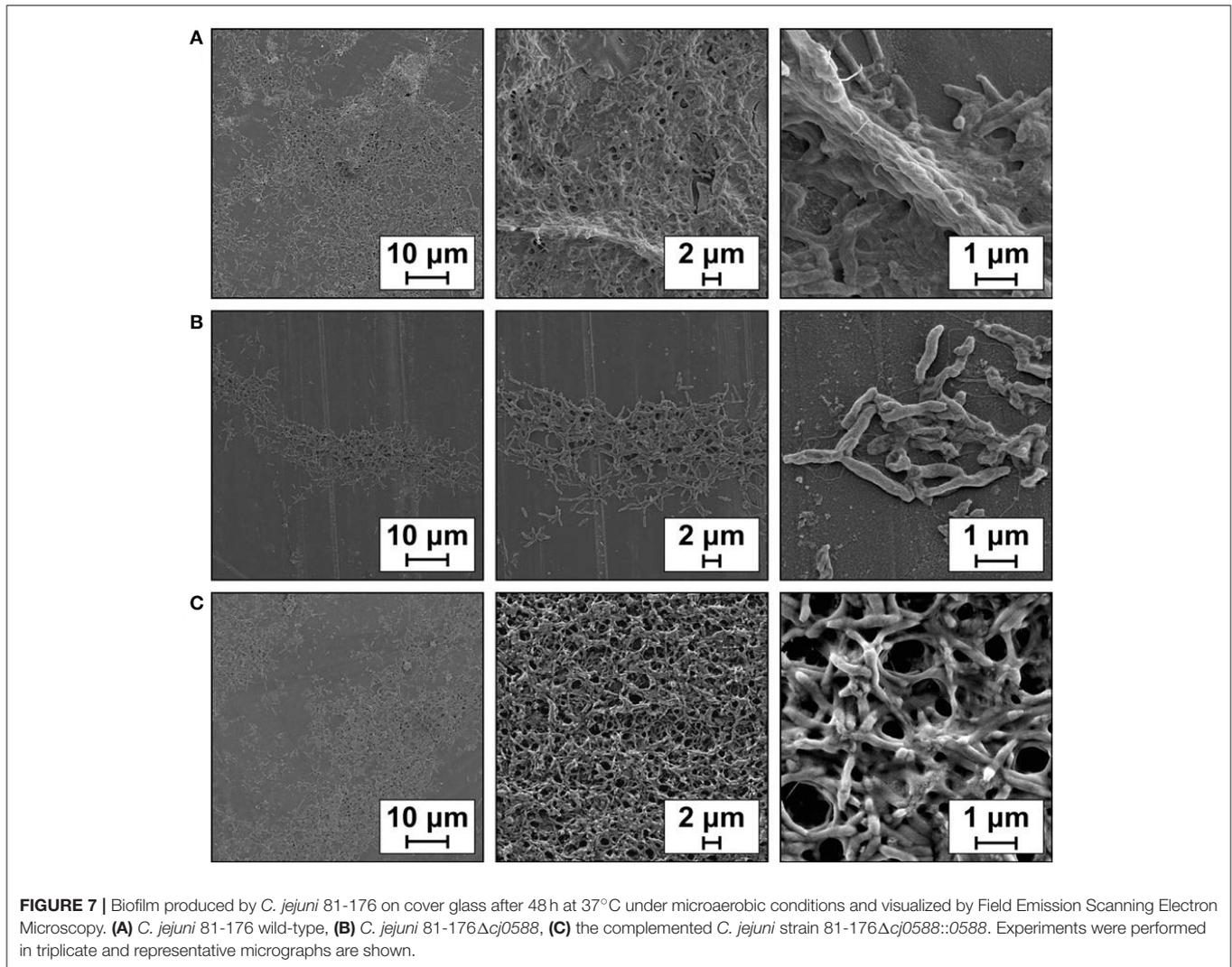


FIGURE 6 | The motility of the *C. jejuni* 405 wild-type strain (WT) was reduced from a growth zone of 54.5 ± 5.4 mm to 19.1 ± 2.7 mm after loss of *cj0588* function ($\Delta cj0588$). Complementation of the null mutant with a plasmid-encoded, active copy of the *cj0588* gene ($\Delta cj0588$ +*cj0588*) partially restored motility to 59% (32 ± 1.2 mm). However, complementation of the null-strain with the K80A, D162A and K188A mutant variants did not rescue this function. The *C. jejuni* 81-176 strain showed the same dependence on Cj0588 activity for motility. The 81-176 strain was generally less motile under these conditions, with a wild-type growth zone of 24.3 ± 8.6 mm, reduced to 8.7 ± 4.2 mm for the *cj0588*-null strain. Complementation by inserting an active *cj0588* gene into the chromosome of the 81-176 null strain restored motility to 66% (16.1 ± 3.5 mm), whereas inactive gene copies did not rescue the phenotype. Values represent means ± S.E.M. of three independent experiments. $P < 0.001$ for WT vs. $\Delta cj0588$; and $P < 0.01$ for $\Delta cj0588$ vs. $\Delta cj0588$ +*cj0588*.



not accompanied by significant slowing of cell growth under the conditions assayed here (Figure S3). The distribution of ribosome particles was rescued by complementation of the null strains with a functional *cj0588* gene. However, complementation with the K80A, D162A, and K188A mutant variants of *cj0588* did not restore the ribosome profiles (Figure 5), confirming that *Cj0588* methylation facilitates the association of the ribosomal subunits.

Inactivation of *Cj0588* Hinders *C. jejuni* Motility

Inactivation of *cj0588* in both the *C. jejuni* 405 and 81-176 strains reduced motility by about 65%, which is consistent with our earlier observations for the 81-176 strain (Salamaszyńska-Guz et al., 2014). We continued the motility studies using the 405 strain after establishing that its growth zones on agar were greater than those of the 81-176 strain (Figure 6). Complementation of the 405-null strain with an active copy of *cj0588* partially restored motility, whereas complementation with the methyltransferase

deficient K80A, D162A and K188A variants did not significantly rescue the *cj0588*-null phenotype (Figure 6).

Biofilm Formation by *C. jejuni* Is Impaired after Inactivation of *cj0588*

Campylobacter jejuni forms biofilms on abiotic surfaces such as polystyrene or glass (Joshua et al., 2006; Kalmokoff et al., 2006; Reeser et al., 2007) as well as on living tissue (Epps et al., 2013; Luethy et al., 2017). The *C. jejuni* 81-176 strain is particularly proficient at attaching to abiotic surfaces (Turonova et al., 2015) in addition to forming aggregates in liquid and biofilms in the human intestinal tract (Joshua et al., 2006; Gunther and Chen, 2009; Haddock et al., 2010). In crystal violet staining assays *in vitro*, the *C. jejuni* 81-176 strain was found to be significantly more effective than strain 405 at forming biofilms on both polystyrene ($A_{570} = 0.53 \pm 0.17$ vs. 0.26 ± 0.05) and on glass. Our subsequent biofilm studies thus focused on the 81-176 strain.

A clear visualization of biofilm formation was attained using Field Emission Scanning Electron Microscopy (FESEM) and

showed that wild-type *C. jejuni* 81-176 cells adhered together to cover the glass almost entirely after 48 h growth (Figure 7A). In contrast, the biofilm formed by the *cj0588*-null strain (Figure 7B) was less extensive, with cells forming clumps lacking the structural roughness of the wild-type strain. These morphological differences were more evident in the enlarged views of individual cells (Figure 8) where the null-mutant cells are clearly less bulky, which would indicate that they have secreted reduced quantities of the extracellular matrix required for biofilm formation. Apart from this difference, the mutant cell images revealed no other obvious variation in dimensions, shape or flagellar structure that might relate to their reduced motility and capacity to form biofilms (Figure 8).

The biofilm forming capacity of the null-mutant was partially restored after being complemented with a functional copy of the *cj0588* gene (Figure 7C), and this correlated with cells regained their bulky appearance (Figure 8C). The inactive versions of the *cj0588* gene did not rescue these morphological features, and cells continued to produce biofilms that were less copious than the null-mutant complemented with the active *cj0588* gene (Figure S4).

DISCUSSION

We show here that the *cj0588* gene of *C. jejuni* encodes a type I TlyA methyltransferase that specifically targets the 2'-*O*-ribose position of 23S rRNA nucleotide C1920, but does not modify 16S rRNA nucleotide C1409. This puts the *C. jejuni* Cj0588 enzyme in the same class as other TlyA^I methyltransferases from *B. hyodysenteriae* and *T. thermophilus*, and distinguishes it from the TlyA^{II} enzymes of *Mycobacterium* and *Streptomyces* species that methylate the ribose of 16S rRNA nucleotide C1409 and also at C1920 (Monshupanee et al., 2012). Both types of TlyA enzyme belong to the cluster of orthologous groups COG2933 and are *S*-adenosylmethionine-dependent, Rossmann-fold methyltransferases related to small RNA-guided fibrillarins found in the Archaea and Eukaryota (Feder et al., 2003). These methyltransferases are characterized by a catalytic tetrad of sequence motifs centered around the conserved amino acid residues K-D-K-E, where three of these residues correspond to K⁸⁰, D¹⁶² or K¹⁸⁸ in the *C. jejuni* Cj0588 enzyme (Figure 1).

Substitution of the equivalent lysine residues in the *E. coli* methyltransferase RrmJ significantly decreased its ability to 2'-*O*-methylate 23S rRNA nucleotide U2552 without affecting the K_m for the enzyme-substrate interaction (Hager et al., 2002). Similarly, each of the K80A, D162A and K188A substitution in Cj0588 resulted in complete loss of methylation at the target nucleotide C1920 in *C. jejuni* (Figure 2, Figure S3). Assays with recombinant Cj0588 proteins that were expressed and purified from *E. coli* showed that cofactor binding was not affected by the K80A, D162A and K188A substitutions and that there was only a moderate reduction in substrate binding (Figure 4). These observations indicate that the substitutions removed the methyltransferase activity of Cj0588 without interfering with its synthesis or its overall tertiary structure. These constructions enabled us to differentiate between effects

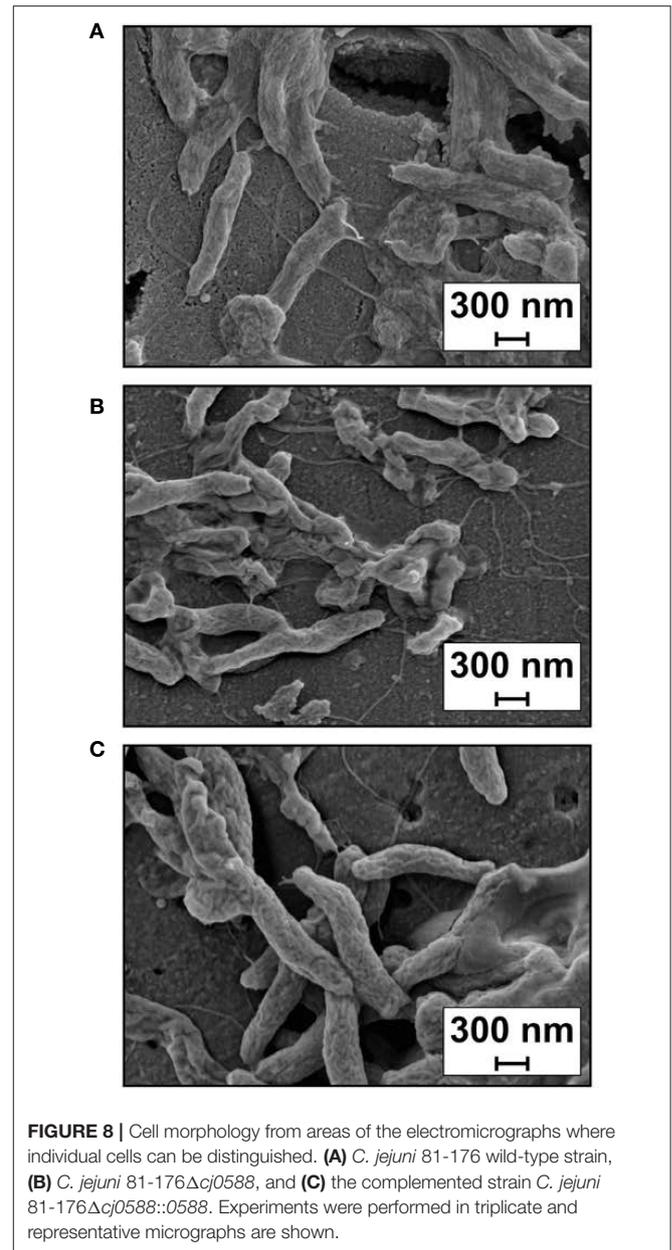


FIGURE 8 | Cell morphology from areas of the electromicrographs where individual cells can be distinguished. (A) *C. jejuni* 81-176 wild-type strain, (B) *C. jejuni* 81-176Δ*cj0588*, and (C) the complemented strain *C. jejuni* 81-176Δ*cj0588*::0588. Experiments were performed in triplicate and representative micrographs are shown.

caused by Cj0588/TlyA-directed rRNA methylation and other putative functions in bacterial virulence that this enzyme might have.

The Cj0588 protein was indeed shown to be linked with an array of additional functions. Loss of Cj0588 reduces the ability of the ribosomal subunit to form a stable 70S complex (Figure 5), increases tolerance to the cyclic peptide antibiotic capreomycin (Figure 5), reduces motility (Figure 6), and diminishes biofilm formation (Figure 7) while decreasing cell bulkiness (Figure 8). These properties are to a large extent restored to wild-type levels by complementing the *C. jejuni* null-strains with an active copy of *cj0588*, and can thus be concluded to be dependent on the presence of the Cj0588 protein.

The more specific question of whether the rRNA methylation capability of Cj0588 promotes these effects was addressed by further rounds of null-strain complementation with the methyltransferase-inactive K80A, D162A, and K188A constructs. For the most part, the conclusions are clear: the phenotypic changes are caused primarily by loss of rRNA methylation and could not be rescued by the catalytically inactive variants of Cj0588. Formation of stable 70S ribosomes and maintenance of capreomycin susceptibility require rRNA methylation. The 23S rRNA nucleotide C1920 is located at the interface where the ribosomal subunits make contact at interbridge B2a (Yusupov et al., 2001; Liu and Fredrick, 2016), which also encompasses the capreomycin binding site (Johansen et al., 2006; Stanley et al., 2010), and C1920 methylation directly affects these processes. Efficient motility of the *C. jejuni* strains also required Cj0588 methyltransferase activity and motility was not restored to wild-type levels by the catalytically defective versions of this protein. These effects thus result from a change in the rRNA methylation at the ribosomal subunit interface, with reduced motility being one of the consequences of altered ribosome function and protein synthesis.

The ability to form biofilms is a defining feature of *C. jejuni* virulence with surface attachment being an essential step in intestinal colonization and infection (Haddock et al., 2010; Epps et al., 2013). It has previously been shown that mutation in the *cj0588/tlyA* gene of *C. jejuni* reduces its colonization of the Caco-2 epithelial cell line (Salamaszyńska-Guz and Klimuszko, 2008). Furthermore, mutation of the *tlyA* homolog in *B. hyodysenteriae* reduces virulence, while loss of *tlyA* function in *Helicobacter pylori* lowers adhesion to human gastric adenocarcinoma cells and prevents colonization of the gastric mucosa of mice (Hyatt et al., 1994; Martino et al., 2001; Zhang et al., 2002). Consistent with these previous observations, we show here that loss of its Cj0588 enzyme results in a significant reduction in the ability of *C. jejuni* to adhere to surfaces *in vitro* (Figure 7). A degree of rescue of the *C. jejuni* biofilm phenotype was obtained by complementation with an active *cj0588* gene, and improvement of the phenotype was clearly better than complementation with inactivated copies of the gene (Figure S4).

Enigmatically, biofilm formation by *C. jejuni* requires flagella expression (Guerry, 2007; Svensson et al., 2014) although motile strains do not necessarily form biofilms (Brown et al., 2015). All the *C. jejuni* *cj0588*-null strains studied here possess flagella (Figure 8) and could to some degree adhere to surfaces (Figure 7B, Figure S4). The *C. jejuni* 405 adhered less well than the slower 81-176 strain to plastic and glass surfaces, consistent with traits other than motility being crucial for biofilm formation. The methyltransferase-deficient cells were less bulky (Figure 8) and tended to form clumps without developing into

the biofilm structures observed for wild type cells and the *cj0588*-complemented cells (Figure 7). In these latter cells, the biofilm phenotype was not fully rescued and the reason for this is not clear. Possibly, removal of *cj0588* and its reintroduction at another chromosomal location could effect changes in the expression of other genes connected with biofilm formation.

Loss of other rRNA modifications have previously been noted to cause a series of pleiotropic effects and result in loss of virulence in pathogenic bacteria (Sergieiev et al., 2011). We show here that loss of the TlyA enzyme homolog Cj0588 in *C. jejuni*, results in defective ribosome subunit association, reduced motility and biofilm formation, and altered sensitivity to the antibiotic capreomycin. Complementation with methyltransferase-defective variants of Cj0588 show that all these effects are related to loss of rRNA methylation rather than loss of the protein itself. From this, it can be concluded that the manner in which Cj0588 is connected with the physiology or pathogenicity of *C. jejuni* is solely a consequence of its rRNA methylation activity. Changes in subunit association and capreomycin sensitivity would result directly from altered ribosomal interactions at the site of the (missing) methylation (Johansen et al., 2006). The phenotypic defects including reduced motility and biofilm formation would result from changes in protein expression on ribosomes lacking the nucleotide C1920 methylation. The exact nature of the proteome changes and how these are linked to loss of motility, biofilm formation and virulence remain to be determined.

AUTHOR CONTRIBUTIONS

AS-G, SR, CL, PB, BT, and TU: Methodology and Investigation; SD and AS-G: Writing, Review and Editing; SD, PB, and AS-G: Resources; SD and AS-G: Funding Acquisition and Supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2017.00533/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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