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The Cj0588 protein is a *Campylobacter jejuni* RNA methyltransferase

Agnieszka Sałamaszyńska-Guz [a,*], Bartłomiej Taciak [b], Agnieszka Kwiatek [c], Danuta Klimuszko [a]

[a] Division of Microbiology, Department of Pre-Clinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Live Sciences – SGGW, Ciszewskiego 8, 02-786 Warsaw, Poland
[b] Division of Physiology, Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Live Sciences – SGGW, Nowoursynowska 159, 02-776 Warsaw, Poland
[c] Department of Virology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

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ABSTRACT

TlyA proteins belong to 2′-O-methyltransferases. Methylation is a common posttranscriptional RNA modification. The *Campylobacter jejuni* Cj0588 protein belongs to the TlyA^{I} protein family and is a rRNA methyltransferase. Methylation of ribosomal RNA catalyzed by Cj0588 appears to have an impact on the biology of the cell. Presence of the *cj0588* gene in bacteria appears to be important for ribosome stability and virulence properties. Absence of the Cj0588 protein causes accumulation of the 50S ribosomal subunits, reduction in the amount of functional 70S ribosomes and confers increase resistance to capreomycin.

1. Introduction

The 2′-O-methylation of ribose is one of the most frequent types of RNA modification. Post-transcriptional modifications of rRNAs influence subunit assembly, conformation changes during protein synthesis and resistance to many ribosome-targeting antibiotics. Drug resistance is conferred by loss or acquisition of rRNA methylation [1–3].

Several families of methyltransferases that modify 2′-hydroxyl groups in ribose have been identified to possess a putative catalytic tetrad K-D-K-E. The K-D-K-E tetrad was reported by Feder et al. [4] also in the TlyA family of RNA methyltransferases. TlyA proteins were found in *Mycobacterium* sp., *Thermus thermophilus* and *Brachyspira hyodysenteriae*; yet, many bacterial genera lack the *tlyA* gene (for example *Escherichia coli*) [3,5]. TlyA orthologues were segregated into two groups: (i) TlyA^{I} that 2′-*O*-methylates nucleotide C1920 in 23S rRNA and (ii) TlyA^{II} that apart from C1920 in 23S rRNA methylates also C1409 in 16S rRNA. Differentiation of these two protein groups is based on the length of N- and C-termini of TlyA^{I} and TlyA^{II} proteins [5]. Moreover, methylation of ribosomes by TlyA proteins causes sensitivity of bacteria to capreomycin and viomycin [3,5,6].

The *Campylobacter jejuni* Cj0588 protein is an orthologue of TlyA proteins. Our previous studies indicate that mutation in the *cj0588* gene influences the adherence abilities of *C. jejuni* to the Caco-2 cell line. This mutation reduces both adhesion and internalization of *C. jejuni* 81-176 and 81116 strains into the epithelial cell line [7]. Studies performed using homologs of the *cj0588* gene – *tlyA*, showed that mutation of the gene affects the colonizing abilities of other pathogenic bacteria, *B. hyodysenteriae* and *Helicobacter pylori* [8–10]. Interestingly, not every *Campylobacter* strain encodes the *cj0588* gene. Prevalence of the *cj0588* gene was determined among the population of Polish *C. jejuni* ($n = 74$) and *Campylobacter coli* ($n = 15$) isolates from children, chickens, pigs and dogs. PCR analysis revealed that all of the *Campylobacter* strains isolated from children carried this marker. For dog and chicken isolates, a similar occurrence (87.5% and 72.7%, respectively) of these genes was noted. Only 7.1% of pig isolates possessed this gene [11].

The aim of the presented work was to determine whether the Cj0588 protein is a methyltransferase and to study the growth properties, polysome profile and antibiotic sensitivity of the *C. jejuni Cj0588*-deficient strain.

2. Material and methods

2.1. Bacterial strains, plasmids, media and growth conditions

Bacterial strains used in this study: *C. jejuni* 81-176 (wild type), *C. jejuni* 81-176 ΔCj0588 (*cj0588* deletion mutant, Km^{r}) [7] and *E. coli* BL21(DE3) – pET588 – strain carrying pET28a with *cj0588* gene coding sequence – pET0588 [7].

C. jejuni strains were grown under microaerobic conditions at 37 or 42 °C on MH agar containing 5% (v/v) sheep blood or MH (bioMerieux). *E. coli* strains were grown at 37 °C in LB broth or on LB agar (bioMerieux) supplemented with km (25 μg/ml).

* Corresponding author. Fax: +48 22 593 60 66.
E-mail address: agnieszka_salamaszynska_guz@sggw.pl (A. Sałamaszyńska-Guz).

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2.2. Spot assay

C. jejuni strains were grown to OD_{600} 1.0 and diluted to a series of concentrations (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). Three microliters of each dilution were dropped on MH agar containing 5% (v/v) sheep blood or MH agar containing 5% (v/v) sheep blood and increasing concentrations of capreomycin (16, 32, 48, 64, 80, 96, 128, 160, 192 and 256 μg/ml) and incubated under microaerobic conditions for 48 h at 37 °C.

2.3. MIC determinations

Overnight cultures of *C. jejuni* strains were diluted to 0.5 McFarland and plated on MH agar containing sheep blood and increasing concentrations of capreomycin (0, 16, 32, 48, 64, 82, 96, 128, 160, 192 and 256 μg/ml). MIC values were defined as the lowest concentration at which no growth was observed after incubation under microaerobic conditions for 48 h at 37 °C.

2.4. Motility assay

Three microliters of the *C. jejuni* culture at OD_{600} 0.1 were spotted onto MH medium with 0.4% agar, left to dry and incubated under microaerobic conditions at 37 °C for 48 h.

2.5. Purification of the Cj0588 protein

Purification of the Cj0588 protein was performed according to Sałamaszyńska-Guz and Klimuszko [7].

2.6. Preparation of ribosomal subunits

Ribosomal subunits of *E. coli* BL21 (*E. coli* lacks *tlyA* gene) were prepared on sucrose gradients as described previously by Douthwaite et al. [12].

2.7. Analytical polysome profiles

Polysome profiles of *C. jejuni* 81-176 wild-type, *C. jejuni* 81-176 ΔCj0588 deletion strain and *E. coli* BL21(DE3) – pET588 strain after induction with 1 mM IPTG were obtained by sucrose gradient centrifugation of the lysates under associating, non stringent salt conditions (20 mM Tris–HCl pH 7.5; 60 mM NH_4Cl, 10.5 mM $Mg(CH_3COO)_2$; 0.1 mM EDTA, 2 mM β-mercaptoethanol). Gradient fractions were monitored with a fractionator (Pharmacia LKB Biotechnology).

2.8. Methylation assay

For the methylation assay the purified Cj0588 protein was used at concentrations of 250 and 500 nM in 50 mM Tris–Cl; pH 7.5; 10 mM EDTA; 10 mM β-mercaptoethanol; 25 mM NaCl buffer. To determine K_m for AdoMet methylation, reactions were carried out in the presence of 6 μM 50S ribosomal subunits and increasing amounts of radioactive [^{3}H]*S*-adenosyl-L-methionine (10.0 Ci $mmol^{-1} = 3.7 \times 10^{11}$ Bq $mmol^{-1}$; Amersham Biosciences). To determine K_m for 50S ribosomal subunits, the methylation assay was performed in the presence of 10 μM AdoMet and varying concentrations of 50S ribosomal subunits. The methylation reactions were performed for 30 min at 37 °C. The reaction was terminated and the mixture was spotted onto 20× 20 mm DE81 filter paper discs (Whatman, Brentford, UK). The filters were air-dried and then washed three times (10 min each) in a large volume of 50 mM KH_2PO_4, once in pure water, and once in 70% ethanol.

23S rRNA was extracted from 50S subunits with phenol/chloroform extraction and analyzed on a 3.5% non-denaturing polyacrylamide gel. Bands representing 23S rRNA were cut from the gel and rRNA washed from the gel using 0.5 M CH_3COONH_4 and 1 mM EDTA buffer.

Incorporation of the ^{3}H-labeled methyl group was measured using a liquid scintillation counter (Wallace, Pharmacia). For kinetic analysis Orgin software was used (OrginLab Corporation).

2.9. Protein 3D-structure prediction

The structural model of the *C. jejuni* Cj0588 protein was obtained based on its amino acid sequence using the SWISS MODEL prediction server [13]. As a template for homology modeling putative hemolysin from *Streptococcus thermophilus* (PDB ID: 3HP7 chain A, at 1.53 Å resolution) was used. Selection was based on results of the FFAS server [14]. To evaluate the degree of accuracy of the obtained model analyses using ProSA-web [15,16] and ProQ [17] were performed. Structure refinement and minimization were carried out using the UCSF CHIMERA program [18]. The structural model was stored on the Protein Model Database (PMDB: PM0078020, http://mi.caspur.it/PMDB/).

3. Results and discussion

3.1. In silico analysis of the C. jejuni TlyA protein sequence

Bioinformatics studies revealed that the Cj0588 protein contains motifs that align well with an rRNA methyltransferase. This enzyme is composed of two protein domains: S4 and FtsJ. Starting at the N-terminus, the S4 domain consists of 64 amino acids and is probably responsible for the initial interaction with the ribonucleic acid, or with a complex of ribonucleic acid and ribosomal proteins, which allows forming a stable enzyme–substrate complex and carrying out the methylation reaction. The second domain, FtsJ, contains four residues: K^{80}-D^{162}-K^{188}-E^{245}, corresponding to the K-D-K-E tetrad in *E. coli* RrmJ (FtsJ) methyltransferase responsible for 2′-O methylation of U2552 in the A loop of 23S rRNA (Fig. 1A and B) [19]. This part of the protein is responsible for the methylation of ribonucleic acids using S-adenosylmethionine as the methyl group donor.

The Cj0588 protein belongs to the $TlyA^I$ group. It lacks four N – terminal amino acids (A2-R3-R4-A5) and has about twenty amino acids shorter C – terminus (Fig. 1A), it suggests that $TlyA^I$ can methylate the 23S rRNA nucleotides.

In silico three-dimensional modeling of the Cj0588 protein showed a structure typical for RNA methyltransferases. The smaller S4 domain covers the active site of the enzyme, which is located in the middle of the FtsJ domain. A characteristic feature is the arrangement of α-helices and β-sheets in the FtsJ domain, constituted by 7 β-sheets surrounded by 5 α-helices – a layout very typical for RNA methyltransferases. Similar arrangement occurs in the larger domain of the *Mycobacterium tuberculosis* TlyA protein and the *E. coli* FtsJ protein (Fig. 1B) [20].

3.2. In vitro methylation activity of the Cj0588 protein

The *in silico* analysis of the Cj0588 amino acids sequence revealed that the protein belongs to the $TlyA^I$ group, and modifies 23S rRNA in 50S ribosomal subunit. Our experimental data confirmed results obtained from *in silico* analysis.

We examined the incorporation of the radioactive methyl group of S-adenosylmethionine into the ribosomal subunits by the Cj0588 protein. First, as substrate for the Cj0588 recombinant protein we used crude ribosomes (called in this paper the S30 fraction) in the presence of radioactively labeled AdoMet as the methyl group donor. The level of ribosome methylation in the presence

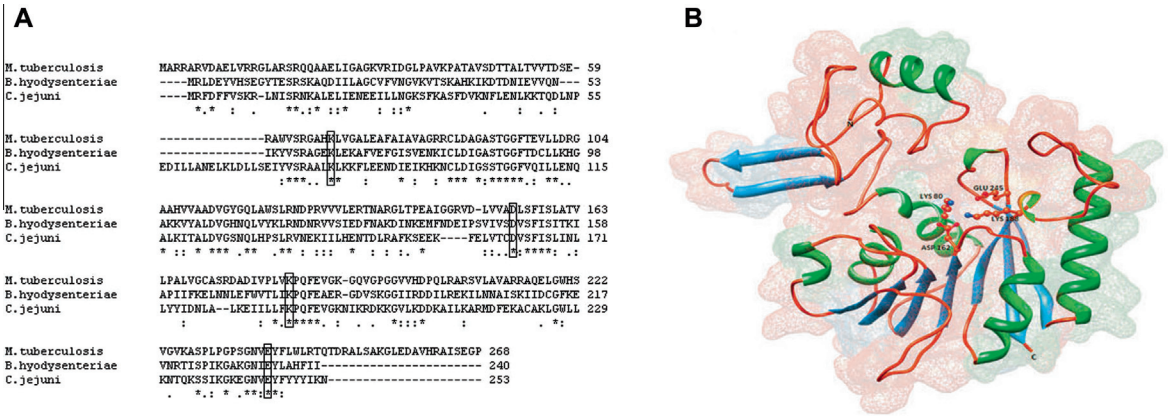

Fig. 1. (A) Amino acid sequence alignment of TlyA from *M. tuberculosis* (ATCC 25618) represents TlyA^{I} group, *B. hyodysenteriae* (B204) represents TlyA^{II} group and Cj0588 from *C. jejuni* (NCTC11168). The catalytic tetrad is showed in boxes. (B) Diagram of the Cj0588 protein tertiary fold.

of Cj0588 was about 40% higher in comparison to the crude S30 fraction (Fig. 2A).

A subsequent step of our experiments was purification of 50S and 30S ribosome subunits from *E. coli*, which has no natural *tlyA* orthologue. These purified ribosome subunits were used as substrates for the Cj0588 protein in the presence of the methyl group donor (AdoMet). As shown in Fig. 2A 500 nM Cj0588 was able to incorporate more methyl groups per 1 pmol of 50S ribosomal subunits in 30 min, compared with incorporation of methyl groups per 1 pmol of 30S ribosomal subunits. Extraction of rRNA from 50S ribosomal subunit and analysis of the methylation products on a polyacrylamide gel revealed that Cj0588 protein methylates 23S RNA in 50S ribosomal subunits (Fig 2B).

C. jejuni grows at temperatures ranging from 30 to 47 °C and therefore is capable of growth at the body temperatures of human and avian hosts, 37 and 42 °C, respectively [21]. Comparison of the enzymatic activity at these two temperatures (37 and 42 °C) showed that the Cj0588 protein demonstrates higher activity at 37 than 42 °C. At 37 °C the enzyme methylates the 50S ribosomal subunit of the 30S fraction and purified 50S subunit (Fig. 2C). In contrast, at 42 °C the enzyme shows reduced methylation of the 50S ribosome subunit of the 30S fraction containing soluble proteins, while almost completely loses methylation activity in the presence of the 50S purified subunit (Fig. 2C).

We characterized the enzymatic properties of the wild-type Cj0588 protein. The V_{max} of the reaction as well as the K_m values for 50S ribosomal subunits and AdoMet were determined by *in vitro* methylation assays using purified Cj0588 and radioactively labeled AdoMet.

To determine the K_m value for 50S ribosomal subunit, saturating concentration of AdoMet (10 μM) and varying concentrations of 50S ribosomal subunits were used. The K_m value for 50S ribosomal subunit was determined to be 4.8 ± 0.6 μM and k_{cat} 0.0048 min^{-1}. To determine the K_m value for AdoMet, the 50S ribosomal subunits were methylated at fixed, saturating concentrations (6 μM) with varying concentrations of AdoMet. The K_m for AdoMet was 5.8 ± 0.6 μM and k_{cat} 0.0044 min^{-1}. K_{cat} values determined for both substrates of the 50S ribosomal subunit and the AdoMet correlated relatively well (varying <20%), indicating that the kinetic constants were obtained under appropriate conditions.

Kinetics of the reaction catalyzed by Cj0588 have shown that the enzyme has higher affinity to 50S subunit than to AdoMet; yet, the K_m values for 50S subunit and AdoMet are at a similar level. Both Cj0588 K_m values are high and exceed the K_m values for *E. coli* FtsJ (RrmJ) reported Hager et al. [22]. For instance, Cj0588 has higher K_m for AdoMet than RrmJ (5.8 μM and 3.7 μM, respectively). The Cj0588 K_m for 50S is 4.8 μM, which is significantly higher than the value determined for the RrmJ enzyme (K_m = 0.8 μM). k_{cat} for the Cj0588 enzyme has been determined to be 0.0044, whereas k_{cat} for FtsJ is 0.064 min^{-1} [22]. Cj0588 has also a significantly lower turnover number compared to the FtsJ protein. Difference in K_m values and turnover number for Cj0588 and FtsJ enzymes could result from the fact that despite both proteins are rRNA methyltransferases and contain a similar catalytic tetrad they methylate

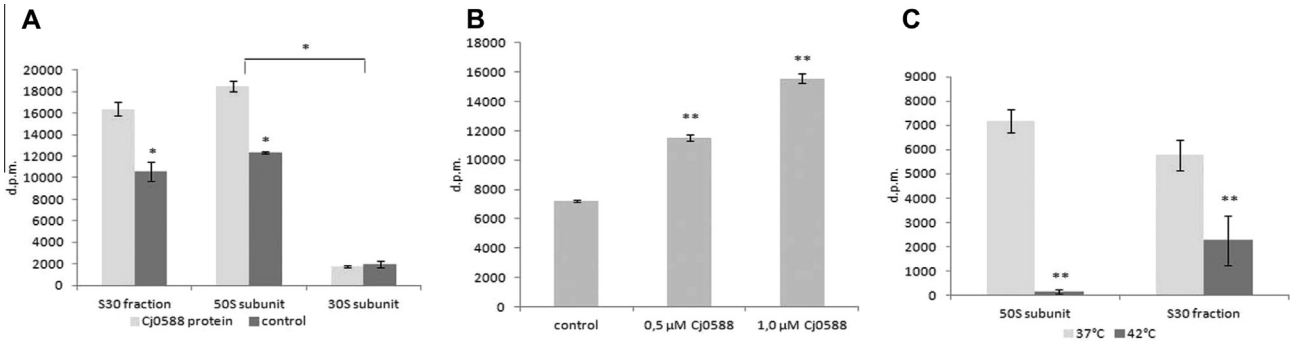

Fig. 2. *In vitro* methylation activity of the purified Cj0588 protein on isolated ribosomes. (A) Methylation of *E. coli* ribosomes in the presence or absence (control) of purified Cj0588 and radioactively labeled S-adenosylmethionine (AdoMet). Cj0588 (500 nM) was incubated with 10 μM radioactively labeled AdoMet and 2 μM S30 fraction, and 4 μM 50S or 30S ribosomal subunits. (B) After incubation with radioactively labeled AdoMet nucleic acids from 50S subunit were extracted and analyzed on a 3.5% polyacrylamide gel, band representing 5S rRNA and 23S rRNA were cut, RNA washed and incorporation of the ^{3}H-labeled methyl group measured (C) Cj0588 (250 nM) was incubated with 10 μM radioactively labeled AdoMet and 2 μM S30 fraction or 6 μM 50S ribosomal subunits in 37 and 42 °C. Values represent means ± S.E.M. of three independent experiments. $^{*}P < 0.025$; $^{**}P < 0.01$.

other nucleotides situated in different regions of the 23S rRNA molecule in 50S subunit.

3.3. Analysis of polysome profiles and growth abilities

The polysome profile of *C. jejuni* 81-176 ΔCj0588 strain was studied and compared to the polysome profile of the *C. jejuni* wild type strain. The *C. jejuni* strains were grown in MH broth at 37 and 42 °C, cells were lysed and ribosomes and ribosomal subunits were separated by sucrose gradient centrifugation under associating, nonstringent salt conditions. The distribution of ribosomal subunits in the mutant strain was strikingly different from that found in the wild type strain that was grown at 37 and 42 °C. The *C. jejuni* 81-176 ΔCj0588 strain displays an increased quantity of free 50S subunits and reduced amount of 70S ribosomes compared with the *C. jejuni* 81-176 wild-type strain (Fig. 3A).

Similar results were obtained for *E. coli* mutants deficient in the RrmJ (FtsJ) methyltransferase, and *E. coli* lacking RrmA, responsible for G475 methylation which also revealed an increased dissociation of their ribosomal subunits. Cells accumulated 50S ribosomal subunits rather than entire 70S ribosomes, which resulted in a slower rate of protein synthesis [19,23–25]. Our results demonstrate that inactivation of the *cj0588* gene and loss of 50S ribosomal subunit methylation influences formation of complete functional ribosomes. Our results also suggest that there is a connection between the Cj0588 protein and the assembly of the entire 70S ribosome.

Inactivation of the *cj0588* gene in *C. jejuni* 81-176 Δ588 causes loss about 70% of motility of the strain (23.1 ± 4.4 mm growth zone for wild type strain and 6.2 ± 2.5 mm for mutant in *cj0588* gene; $P < 0.01$) (Fig. 3B); yet, growth abilities are the same like for the *C. jejuni* 81-176 wild-type (Fig. 4 – MH agar). Previously, we have shown that inactivation of the *cj0588* gene reduces adhesion of *C. jejuni* 81-176 and 81116 strains to epithelial Caco-2 cells [7]. Reduction of adhesion abilities by the *C. jejuni* 81-176 ΔCj0588 cells is consistent with the observation of reduced motility.

Most translational apparatus modifications made by bacterial enzymes, besides functional conformational changes, confer also resistance to a large group of antibiotics that target ribosomes. Such modifications seem to be logical in the sense of evolution, where bacteria by amending in their molecular structure, resistance against selection factors. Yet, this simple explanation does not necessarily apply to the bacterial TlyA proteins. The methyl group incorporated to the ribonucleic acid confers susceptibility to antibiotics; yet, at the same time such modification, as mentioned above, is necessary for ribosome assembly and thus proper functioning of the cell.

We tested the effects of capreomycin which interacts with ribosomes on *C. jejuni* cells. *C. jejuni* 81-176 and *C. jejuni* 81-176 Δ588 strain were growth on MH agar and increasing concentrations of capreomycin and growth abilities of strains were tested (Fig. 4). The MIC value of capreomycin for *C. jejuni* 81-176 was 64 μg/ml. For *C. jejuni* 81-176 ΔCj0588, the MIC value increased 2-fold to 128 μg/ml.

Loss of rRNA methylation catalyzed by the Cj0588 protein resulted in a 2-fold increase of MIC of capreomycin against *C. jejuni* 81-176 ΔCj0588 cells. Similar results were obtained by Maus et al. [6], who demonstrated that mutation of the *tlyA* gene in *M. tuberculosis* cells also increased resistance to capreomycin. Moreover, analogous results were reported by Monshupanee et al. [5], who studied *E. coli* strains expressing the recombinant *tlyA* gene from *Mycobacterium smegmatis*, *B. hyodysenteriae*, *Streptomyces coelicolor*, *Geobacillus stearothermophilus* and *T. thermophilus* [3,5,6].

To sum up, our studies provide evidence that not only the Cj0588 protein is an rRNA methyltransferase, but also that it is involved in formation of entire ribosomes. Moreover, we demonstrate that some biological functions of rRNA methylation might be catalyzed by Cj0588.

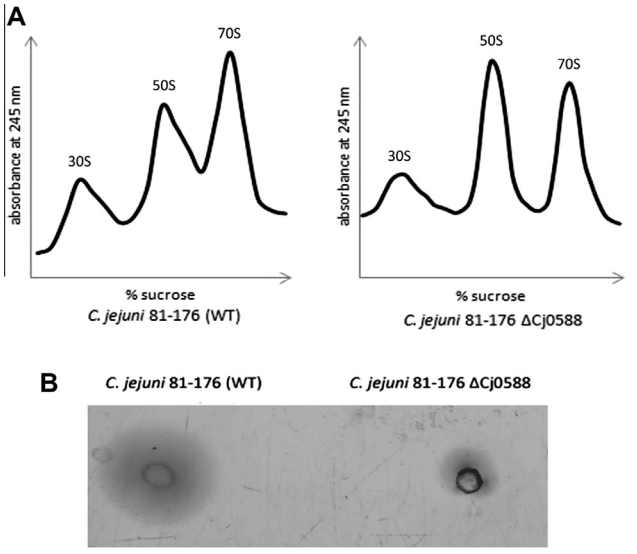

Fig. 3. Phenotypes of *C. jejuni* 81-176 ΔCj0588 and wild-type *C. jejuni* 81-176 strain. (A) Polysome profiles of the cj0588 deletion strain (*C. jejuni* 81-176 ΔCj0588), and wild-type *C. jejuni* 81-176 strain. Sucrose gradient sedimentation profiles of extracts from wild-type *C. jejuni* 81-176. Positions of 30S and 50S ribosomal subunits and 70S monomers are indicated. (B) Motility assay.

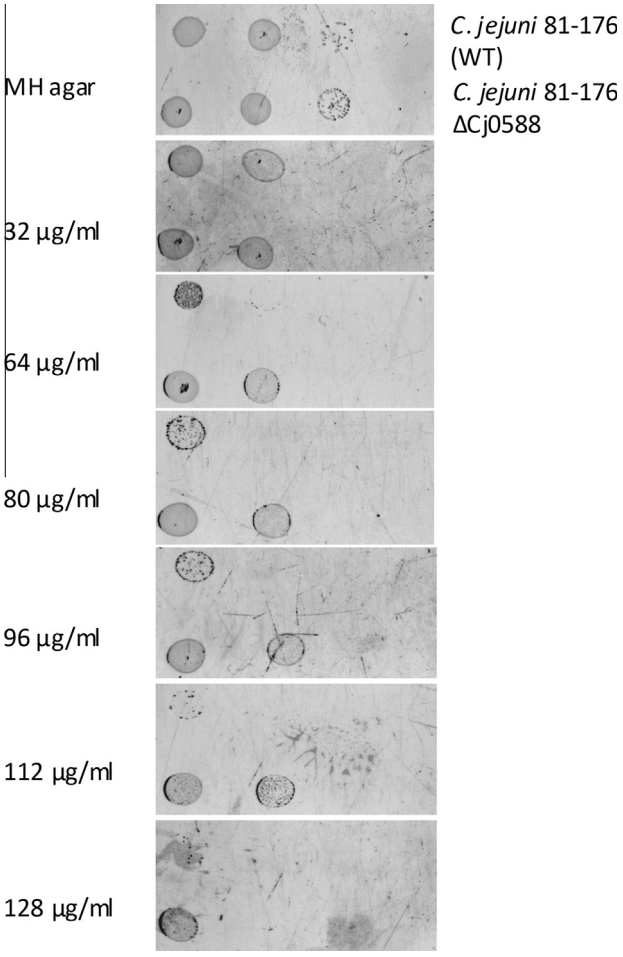

Fig. 4. Spot assay of wild-type *C. jejuni* 81-176 and *C. jejuni* 81-176 ΔCj0588 mutant strain on MH agar plates with capreomycin (32, 64, 80, 96, 112, 128 μg ml^{-1}).

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