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Structural and functional basis for targeting *Campylobacter jejuni* agmatine deiminase to overcome antibiotic resistance

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Abstract

Campylobacter jejuni is the most common bacterial cause of gastroenteritis and a major contributor to infant mortality in the developing world. The increasing incidence of antibiotic-resistant *C. jejuni* only adds to the urgency to develop effective therapies. Because of the essential role that polyamines play, particularly in protection from oxidative stress, enzymes involved in the biosynthesis of these metabolites are emerging as promising antibiotic targets. The recent description of an alternative pathway for polyamine synthesis, distinct from that in human cells, in *C. jejuni*, suggests this pathway could be a target for novel therapies. To that end, we determined X-ray crystal structures of *C. jejuni* agmatine deiminase (CjADI) and demonstrate that loss of CjADI function contributes to antibiotic sensitivity, likely due to polyamine starvation. The structures provide details of key molecular features of the active site of this protein. Comparison of the unliganded structure (2.1 Å resolution) to that of the CjADI-agmatine complex (2.5 Å) reveals significant structural rearrangements that occur upon substrate binding. The shift of two helical regions of the protein and a large conformational change in a loop near the active site generates a narrow binding pocket around the bound substrate. This change optimally positions the substrate for catalysis. In addition, kinetic analysis of this enzyme demonstrates that CjADI is an iminohydrolase that effectively deiminates agmatine. Our data suggest that *C. jejuni* agmatine deiminase is a potentially important target to combat antibiotic resistance, and these results provide a valuable framework to guide future drug development.

TOC image

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The coordinates of structures reported have been deposited in the Protein Data Bank as entries 6B10 for the unliganded CjAD and 6B2W for the CjAD-agmatine complex.

Supporting Information.

The supporting information file contains four supplementary figures that illustrate and provide quantitation of the protein purity (Figure S1), show the conservation of the active site and selectivity for the substrate (Figure S2), illustrate the proposed mechanism catalyzed by CjADI (Figure S3), and provide kinetic support for CjADI selectivity of agmatine over arginine as a substrate (Figure S4).

Keywords

polyamine biosynthesis; agmatine iminohydrolase; antibiotic resistance; N-carbamoylputrescine; putrescine

INTRODUCTION

Campylobacter jejuni is a gram negative bacterium that is one of the most common causes of acute gastroenteritis in the developed world.¹⁻³ *Campylobacter* spp. are also a major contributing factor to childhood morbidity caused by diarrheal illness.^{1, 3} While many patients respond well without medical intervention, antibiotics are often used to treat *Campylobacter* infections, particularly in the case of immunocompromised individuals or when the bacteria invade the intestinal mucosa. Fluoroquinolones, erythromycin and macrolides are commonly used to treat *C. jejuni* infections. Resistance to macrolide and fluoroquinolone antibiotics, however, is now widely reported⁴ and the World Health Organization (WHO) considers fluoroquinolone resistant *C. jejuni* a high priority pathogen.⁵

Exposure to these classes of antibiotics is known to increase reactive oxygen species (ROS) within bacterial cells and enhances bacterial death and structural damage due to oxidative stress.⁶ Bacterial cells respond to ROS by producing polyamines, such as putrescine and spermidine, which act as antioxidants.⁷⁻⁹ Inhibition of polyamine biosynthesis in *E. coli* results in increased oxidative stress and mortality of antibiotic-treated cells.⁸ Furthermore, the addition of exogenous spermidine in *P. aeruginosa* increases the minimal inhibitor concentration (and thus increased resistance) to multiple drug classes, including aminoglycoside and quinolone drugs, and enhanced cell viability during antibiotic challenge in *E. coli* cultures.^{8, 10} Thus, pathways specific for polyamine biosynthesis in bacteria could be considered valuable targets for the development of new antibiotics or as adjuvants to combat antibiotic resistance.

Polyamines, including spermidine, spermine, putrescine, and cadaverine, are essential metabolites found in all kingdoms of life.¹¹ All eukaryotes and many bacteria that synthesize the triamine, spermidine, utilize the enzymes *S*-adenosylmethionine decarboxylase (AdoMetDC) and spermidine synthase (SpdSyn).¹² However, many bacteria known to synthesize spermidine do not contain AdoMetDC or SpdSyn homologs. The *C. jejuni* genome does not contain genes for AdoMetDC or SpdSyn; biosynthesis of polyamines in this organism proceeds by a recently identified alternative aspartate β -semialdehyde pathway (Fig. 1).¹³ The second step in the pathway, the conversion of agmatine to *N*-carbamoyl putrescine, is catalyzed by agmatine deiminase (ADI, also referred to as agmatine iminohydrolase, EC 3.5.3.12). As this enzyme is not present in humans, and the production of spermidine is critical for *C. jejuni* proliferation¹³, the *C. jejuni* ADI (CjADI) is a potential target for the development of novel antibiotics. In addition to its role in bacterial polyamine synthesis, agmatine is an endogenous human cell-signaling molecule that triggers an innate immune response, which could enhance the immune response during infection.^{14, 15} Thus, an ADI inhibitor could reduce *C. jejuni* virulence by causing an accumulation of agmatine, thus enhancing the host immune response, and by increasing oxidative stress in the pathogen via reduction of the cytoplasmic polyamines response.

The gene corresponding to ADI in the genome of *C. jejuni* (Cj0949c) is annotated as a peptidyl-arginine deiminase (PAD; NCBI Gene database). Cj0949c is located in the same operon as Cj0947c, which encodes an *N*-carbamoyl putrescine amidohydrolase (NCP). Thus, similar to the recently characterized *Helicobacter pylori* agmatine deiminase¹⁴, we believe that Cj0949c encodes an agmatine deiminase and does not possess PAD activity.¹⁶ Both agmatine deiminase and peptidyl-arginine deiminase belong to the guanidine-group modifying enzyme (GME) superfamily. Agmatine deiminase catalyzes the conversion of agmatine (a decarboxylation product of arginine) to *N*-carbamoylputrescine and ammonia (Fig 1). Mechanistic studies on agmatine deiminases have identified conserved Asp, His, and Cys residues that are critical for catalysis. The proposed mechanism involves nucleophilic attack by the conserved cysteine and proceeds through the formation of a tetrahedral intermediate.¹⁴

In this work we report crystal structures of both the unliganded wild-type and a catalytic mutant of *C. jejuni* agmatine deiminase (CjADI) with the substrate, agmatine, bound. The structure of the enzymatically inactive C315S mutant with agmatine bound provides molecular-level details of critical active site interactions between protein and substrate. We provide evidence from kinetic assays that CjADI is an agmatine deiminase and not a peptidyl-arginine deiminase. In addition, we determined the efficacy of aminoglycoside antibiotics against an ADI deficient mutant of *C. jejuni* and demonstrate that CjADI mediates *C. jejuni* sensitivity to antibiotics. This work helps to establish CjADI as a feasible target for antibiotic drug development and provides a structural framework for future drug discovery efforts.

MATERIALS AND METHODS

Cloning and site-directed mutagenesis

Wild-type agmatine deiminase from *C. jejuni* and the C315S mutant were expressed from a modified pET-28 vector (pTHT, with a Tobacco Etch Virus protease recognition site in place of the thrombin site) with an N-terminal hexahistidine tag. Standard methods were used for DNA restriction endonuclease digestion, ligation, and transformation.¹⁷ Plasmid DNA was purified with the GeneJet miniprep kit (Fermentas, Glen Burnei, MD). Agarose gel electrophoresis was used to separate DNA fragments. The fragments were excised and purified with the Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA). *E. coli* strain Mach1 (Invitrogen, Madison, WI) was used as a recipient for transformations during plasmid construction. An Eppendorf Mastercycler and Phusion DNA polymerase (New England Biolabs, Ipswich, MA) were used for polymerase chain reactions (PCR). All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). The ADI gene was amplified from *C. jejuni* genomic DNA by PCR with the following primer pair: 5'-GGG TAG CAT ATG ATA AAA TCA ATT CCT GAA TGG AGT G-3' and 5'-CCC TAC TCG AGT CAT CTT AAA CCT TTG AAA CGA TTT TG-3'. This purified PCR product was digested with NdeI and BamHI, purified and ligated into similarly digested pTHT. Colonies were screened for presence of the insert and a representative plasmid was designated pCjADI.THT. The plasmid was sequenced at the Cornell Life Sciences Core Laboratory and shown to contain no errors. The pCjADI.THT clone was used as a template to generate the C315S mutant by site-directed mutagenesis. Mutagenesis was performed by a standard PCR protocol using OneTaq DNA Polymerase (New England Biolabs, Ipswich, MA) and DpnI (New England Biolabs, Ipswich, MA) was used to digest the methylated parental DNA prior to transformation. The forward and reverse primers used were 5'-CGA CAA AAC GGT TCT TTG CAT AGT TCT TGT CAA AAT CG-3' and 5'-CGA TTT TGA CAA GAA CTA TGC AAA GAA CCG TTT TGT CG-3', respectively. The presence of the mutated residue was verified by sequencing (Genscript, Piscataway Township, NJ).

Protein expression and purification

E. coli BL21 (DE3) cells were transformed with pCjADI.THT. Overnight cultures were grown in Luria Broth (LB) media supplemented with 50 µg/mL kanamycin at 37 °C. The resulting cultures were pelleted, and used to inoculate 1 L of LB media supplemented with 50 µg/mL kanamycin. The cells were grown with shaking at 37 °C. When the cells reached an OD₆₀₀ of 0.7-0.8 the temperature was lowered to 15 °C and overexpression of CjADI was induced with the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside. The culture was then left to incubate overnight. Following standard expression and purification methods, an inactive form of CjADI is produced. This is consistent with previous reports for the *Helicobacter pylori* homolog that the agmatine deiminase can be inactivated that metals that copurify during expression and purification.¹⁴ To generate an active form of the enzyme we used a modified protocol. Specifically, at protein induction 100 µM 1,10-phenanthroline was added to sequester metals, followed by the addition of 70 µM MnCl₂.¹⁸ After overnight growth, cells were pelleted by centrifugation at 4 °C for 30 min at 6,000 *g* and then stored at -20 °C until purification. Cells were resuspended in nickel binding buffer (20 mM Tris (pH

8.0), 500 mM NaCl, 30 mM imidazole) and lysed by sonication. The cell lysate was then cleared by centrifugation at 40,000 *g* for 30 min at 4 °C. CjADI was purified from the cell lysate by immobilized metal affinity chromatography. A Ni-NTA column was equilibrated in Ni-NTA binding buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 30 mM imidazole) to which the lysate was added. The column was washed with 100 mL of the nickel binding buffer, and the CjADI was eluted from the column by the addition of nickel binding buffer supplemented with 250 mM imidazole. The sample was dialyzed into a storage buffer consisting of 10 mM Tris, pH 8.0 and 100 mM NaCl and concentrated to 100 μ M before being flash cooled and stored at –80 °C. Based on SDS-PAGE analysis, the resulting CjADI protein was ~88% pure (Fig. S1). The C315S mutant was expressed and purified in the same manner as described above. Note that, for both samples the His-tag was not removed. This results in a protein with the following N-terminal tag: MGSDKIHSHHHSSGENLYFQG.

Protein crystallization and data collection

Initial crystallization conditions were identified with the hanging drop vapor diffusion method using sparse matrix screens (Crystal Screens 1 and 2, PEG/ION and PEG/ION 2, Hampton Research) at 18 °C. Hanging drops consisted of 1 μ L of protein mixed with 1 μ L of mother liquor. Rod-shaped crystals were obtained in a condition containing 0.2 M potassium phosphate, pH 4.6, and 20% polyethylene glycol 3350. Crystals were transferred to a cryoprotectant solution composed of the mother liquor supplemented with 20% ethylene glycol and flash cooled in liquid nitrogen prior to data collection. Attempts at co-crystallization and ligand soaking were conducted using arginine and agmatine, but were unsuccessful with the wild-type protein. Crystals for the C315S mutant were obtained by microseeding with wild-type crystals in 0.2 M potassium chloride and 20% polyethylene glycol 3350, pH 7.0. A ligand bound structure of the C315S was obtained by soaking in the crystallization solution supplemented with 3 mM agmatine and 20% ethylene glycol, prior to freezing. Data collection was conducted at 100 K at the A1 beamline of MacCHESS (unliganded native protein) and 24-ID-E at APS (CjADI-agmatine complex). The data collection statistics are summarized in Table 1.

Structure solution and refinement

In both cases, the data was indexed, integrated, and scaled with XDS (liganded structure)²⁰ and HKL-2000 (unliganded structure).²¹ Structures were solved by molecular replacement using Phaser²² with the *Helicobacter pylori* protein structure (coordinate file 3HVM¹⁴, 45% sequence identity) as the search model. Initial refinement was carried out with the PDB-REDO server.²³ The models were further refined using iterative rounds of manual model building with Coot^{24, 25}, and restrained refinement with Refmac5²⁶. Non-crystallographic symmetry restraints were used for initial rounds of refinement but removed in later stages. After the refinement had converged, water molecules were added using Coot. Following additional rounds of refinement, the ligand was built into a clear region of $F_O - F_C$ density (contoured at 3 σ) and a final round of refinement was conducted. Restraints for the ligand were generated using the PRODRG2 server.²⁷ The data refinement statistics are summarized in Table 1. Note that the his-tag (N-terminal of Glu – 7) was not ordered in either of the structures. Also, in the unliganded structure (6B10), several residues in loop regions in

Chain A were not modeled due to gaps in the density. These include residues 103 – 109, 161 – 164, and 192 – 196. These regions are ordered in the liganded structure (6B2W).

Steady-state kinetic assays

CjADI activity was measured for the wild-type ADI and the C315S variant using a previously described assay that couples ammonia release to nicotinamide adenine dinucleotide (NADH) oxidation.^{28, 29} All kinetic assays were performed at 37 °C, the standard temperature for ADI kinetic assays.¹⁴ The assay buffer consisted of 500 mM Hepes (pH 7.0), 1 mM NADH, 1 mM α -ketoglutarate, 4 units of glutamate dehydrogenase, and varying concentrations of agmatine. Similar experiments were also performed using arginine as the substrate and source of ammonia. The reaction was initiated by the addition of 5 μ M ADI (wild type or variant) and the resulting decrease in absorbance at 340 nm was monitored for every minute for 3 hours in a Synergy HT micro-plate reader (Biotek). To control for the background rate of NADH oxidation, samples were measured in the absence of ADI at the highest substrate concentration and subtracted from the absorbance change. The kinetic parameters k_{cat} , k_{cat}/K_M and K_M were determined by fitting the Michaelis-Menten equation to the initial velocity (less than 10% reaction completion) as a function of substrate concentration. All kinetic data were analyzed using the KaleidaGraph software package (Synergy Software).

Disk diffusion and agar dilution assays

C. jejuni WT (81116) and agmatine deiminase mutant (C8J_0892, kind gift of Bruce M. Pearson)¹³ were grown for 48 hours on standard Mueller-hinton (MH) agar plates at 37 °C under standard microaerophilic growth conditions prior to antibiotic testing (Campy GasPak system, BD biological). Bacteria were suspended in MH broth to OD₆₀₀ of ~0.1 (equivalent to $\sim 1 \times 10^8$ CFU/ml). Sterile swabs were used to spread bacteria over the surface of MH or MHS (Mueller Hinton agar supplemented with spermidine at 500 μ M). Standard disks containing gentamycin (10 μ g), streptomycin (10 μ g) and ciprofloxacin (5 μ g) were placed on the agar surface. After incubation (37 °C, 48 hr, microaerophilic conditions), inhibition of growth was recorded as the diameter (mm) of inhibition halos from three separate agar plates.³⁰ Agar dilutions of streptomycin in two-fold dilutions from 8 μ g/mL stocks in MH and MHS agar were inoculated with 10 μ l of bacteria suspended in MH broth (as a spot on the surface of the plate and left to dry, in duplicate spots per agar plate) containing 1×10^4 CFU of either 81116 or the AIH mutant. After incubation (37 °C, 48 hr, microaerophilic conditions), the growth was observed and MIC determined as the first agar plate lacking colony growth for each strain.³⁰

RESULTS AND DISCUSSION

Structure of CjADI

To better understand the role of the alternative polyamine biosynthetic pathway and to facilitate drug discovery efforts we determined the X-ray crystal structure of the *C. jejuni* agmatine deiminase. CjADI is a 325-amino acid protein with a molecular mass of 37.9 kDa. Crystals of CjADI grew overnight and crystallized in space group $P2_1$ with two molecules observed in the asymmetric unit (~46% solvent content). Unlike homologous ADI enzymes,

such as from *H. pylori*¹⁴, the structure of CjADI is observed as a monomer in the crystal (the largest crystallographic interface surface was 161 Å², as calculated by PISA³¹) and in solution (by size exclusion chromatography, data not shown). As a member of the guanidine-group modifying enzyme, the overall structure of CjADI exhibits the canonical α/β -propeller fold (Fig. 2A).¹⁶ The active site (marked by conserved residues shown in ball-and-stick notation in Fig 2A) resides at the core of the propeller. Comparison of the structure of CjADI to the ADI of *Helicobacter pylori* (3HVM, Fig. 2B) or *Arabidopsis thaliana* (IVKP, Fig. 2C) reveals a high degree of conservation of the overall fold throughout evolution.¹⁴ A number of other structures available in the protein data bank (2CMU, 44% sequence identity to CjADI; 1ZBR, 36% sequence identity; and 1XKN, 35% sequence identity; all from structural genomics groups), annotated as putative peptidyl-arginine deiminases, are almost certainly agmatine deiminases as well (Fig. S2). *CjADI active site and substrate binding.* To characterize the active site and substrate binding interactions of CjADI we determined the crystal structure of the protein in complex with its substrate. All attempts to co-crystallize or soak the ligand, or its structural analog, arginine, into the native protein were unsuccessful, yielding maps without clear ligand density in the active site. This is likely due to the rapid turnover of the substrate. To generate an enzymatically inactive form of the protein we mutated the catalytic cysteine to serine. This C315S mutant crystallized under similar conditions as the native protein and, upon soaking with a cryo-solution containing agmatine, we were able to obtain a structure of CjADI in complex with substrate (Fig. 3).

As expected, the four residues previously observed to be directly involved in the catalytic cycle (C315, D201, D82 and H199)^{14, 32, 33} are all closely clustered around the guanidinium group of agmatine (Figs. 3A, 3B and S2A). The catalytic cysteine (serine in the C315S structure) is well positioned for nucleophilic attack at the carbon atom of the guanidinium and a well ordered water molecule is situated appropriately for the final hydrolytic step of catalysis. In addition, the orientation of the amino acid side chains in the structure of the CjADI-agmatine complex, as well as the position of the substrate, are all consistent with the proposed mechanism (Fig. S3).^{32, 33} Following the numbering of CjADI, H199 would be expected to be the proton donor and acceptor for the leaving group, while the two aspartate residues (D82 and D201) would serve mainly to orient the substrate for catalysis. The guanidinium group of the agmatine undergoes nucleophilic attack by the thiolate of C315, forming a tetrahedral intermediate. After loss of a molecule of ammonia and formation of an S-alkylthiuronium intermediate, a water molecule is deprotonated by H199 to activate it for nucleophilic attack. A second tetrahedral intermediate is then formed, which collapses to form the final product.^{32, 33}

In the highly structurally conserved binding pocket^{14, 34}, there are relatively few hydrogen bond interactions between the protein and the ligand in the active site (Fig. 3). Only H199, Asp201 and T196 are within hydrogen bonding distance of the guanidinium group (Fig. 3B). Instead, several aromatic groups appear to be the predominant contributors to shaping the active site. Two conserved tryptophan residues (W79 and W104) and a conserved phenylalanine residue (F108, conserved as a phenylalanine or tyrosine) partially surround the substrate (Figs. 3). Two additional residues, D77 and Q309, also make hydrogen bond interactions with the terminal NH₂ group of agmatine. The active site resides within a deep pocket of the protein (Figs. 4A and 4B). Only a narrow opening allows access of the

substrate, which requires the guanidinium group enter first (Fig. 4B). This orientation is presumably aided by the presence of several polar groups, including D24, D195, Q309, and N310 that line the entry to the active site (Fig. 4C).

Structural changes upon substrate binding

Initial characterization of the structural flexibility of unliganded CjADI revealed several potentially flexible regions (Fig. 5). Examination of the distribution of B-factors throughout the structure (Fig. 5A) suggests that three loop regions, all located near the active site, may have a high degree of structural instability. Indeed, comparison of the unliganded to the agmatine-bound structure of CjADI shows that the largest structural changes occur at two of these three regions (Fig. 5B). Because of the proximity to the active site of these flexible regions, we speculate that the observed structural changes may be a result of an induced fit upon substrate binding. Examination of the CjADI-agmatine complex shows that significant rearrangements do indeed occur upon substrate binding (Fig. 5C). In addition to slight movement of the tryptophan residues that line the active site (W79 and W104), a large loop movement occurs that brings threonine 196 and aspartate 195 into close proximity to the substrate. This loop, which is relatively disordered in the unliganded structure, becomes much more ordered when the substrate is bound. These observations confirm that CjADI undergoes distinct conformational changes in secondary structural elements upon substrate binding. These changes appear to order the amino acids in the active site into more favorable orientations and distances for substrate interactions, presumably to produce the most productive conditions for efficient catalysis.

Steady state kinetics of CjADI

Initially, we expressed and purified CjADI using standard immobilized metal affinity chromatography methods. Despite successfully obtaining high levels of pure protein, we were unable to observe any measurable activity for the most likely substrates, agmatine or arginine. Prior studies of agmatine deiminase indicate that the protein could be inhibited by various divalent cations.^{14, 35} We, therefore, expressed and purified CjADI using a modified method (see Materials and Methods section) to reduce the free metal concentration, which could inactivate the enzyme. The resulting enzyme from this modified method was active and had a K_M and k_{cat} for agmatine of $70 \pm 16 \mu\text{M}$ and $0.32 \pm 0.02 \text{ min}^{-1}$, respectively (Fig. 6). Mutation of the active site cysteine to a serine (C315S) produced an inactive form of the enzyme, even when purified using our modified method. As a measure of substrate specificity, the ability of CjADI to deiminate arginine was also tested. However, even at 3 mM arginine, no activity was observed over background (Fig. S4). As protein-arginine deiminases have been reported to be dependent upon the presence of calcium^{36–39}, we also tested the arginine deiminase activity in the presence of calcium. Even in the presence of a 10-fold molar excess of calcium, no arginine deiminase activity was observed (Fig. S4). Superposition of arginine onto agmatine in the active site suggests that residues D77, D195, Q309 and N310 confer this substrate selectivity (Fig S2B). These data demonstrate that CjADI is a *bona fide* agmatine deiminase with strong specificity for its native substrate over the structurally related analog, arginine.

Loss of agmatine deiminase function increases *C. jejuni* sensitivity to aminoglycoside antibiotics

The deletion of the ADI-encoding gene in *C. jejuni* (locus 0892 in the *C. jejuni* 81119 genome) has been shown to eliminate the production of spermidine in this organism.¹³ Using a disk-diffusion assay we found that the loss of ADI activity makes *C. jejuni* more sensitive to the aminoglycoside antibiotics gentamicin and streptomycin (Fig.7). This phenotype was observed on MH agar, the standard agar for disk-diffusion antimicrobial testing in bacteria, which is presumably limiting enough in exogenous spermidine to generate spermidine starvation in the mutant. Indeed, addition of 500 μ M spermidine to the MH (MHS agar) restored the resistance phenotype of the CjADI knockout to the wild-type levels for both drugs (Fig. 7). Agar dilution testing revealed that the CjADI knockout strain had a minimal inhibitory concentration (MIC) of 2 μ g/mL streptomycin on MH, compared to 4 μ g/mL for the wild-type strain (81119). On MHS, both strains had an MIC of 4 μ g/mL streptomycin, confirming the observation that the loss of spermidine biosynthesis increased the sensitivity to aminoglycoside antibiotics. While the change in streptomycin MIC on the 81119 background appears minor, *Campylobacter* mutants with very high resistance to aminoglycoside antibiotics are increasingly common.⁴⁰ In those mutants, inhibition or inactivation of spermidine biosynthesis is likely to have a more dramatic effect on the efficacy of aminoglycoside therapy. Reported breakpoints for aminoglycosides in *Campylobacter* indicate that 4 μ g/ml represents intermediate resistance, while an MIC of 2 μ g/mL is considered sensitive⁴¹ to the drug.

The increasing incidence of fluoroquinolone resistance in *C. jejuni* is one of many drivers underlying the current urgency to develop novel or improved antibiotics. In particular, the identification of new targets and mechanisms to combat antibiotic resistance are sorely needed. Enzymes involved in polyamine biosynthesis are potentially favorable targets for the development of new antibiotics. Bacteria require polyamines for growth; these molecules are involved in controlling chemotaxis⁴², biofilm formation⁴³, resistance to nitrosative stress⁴⁴, and can play roles in virulence and drug resistance.^{10, 45, 46} Notably, many intracellular pathogens have a defined need for spermidine during infection, with a strong correlation between virulence and the reduction of oxidative or nitrosative stress by polyamines.^{46–48} In addition, since polyamines are positively charged, tight control over cellular concentration of these molecules is critical. Concentrations of polyamines that are too high can have toxic deleterious effects, such as precipitating DNA through ionic interactions.^{49, 50}

Prior studies^{7–10}, and the data presented above, suggest that knock-down of the agmatine deiminase in *C. jejuni* would increase oxidative stress in the pathogen and reduce resistance to antibiotic treatment. This suggests that inhibiting CjADI might be a viable strategy to improve the efficacy of existing antibiotics and to reduce the incidence of resistance. The agmatine deiminase from *C. jejuni* belongs to an alternative, evolutionarily divergent, spermidine biosynthetic pathway. This divergence contributes to a greater potential for specificity in drug development. In addition, known mechanisms of antibiotic resistance in *C. jejuni* generally involve horizontal gene transfer events, which provide the cells the means to alter the structure of, and thus inactivate, the aminoglycosides⁴. These mechanisms would not be expected to alter the efficacy of drugs that target polyamine biosynthesis. Thus,

CjADI is a potentially important target for adjunct therapy without a demonstrated selective pressure for mutation during aminoglycoside therapy. The structural and functional characterization of CjADI detailed herein, will serve to accelerate progress towards this end. These results furnish valuable insights into the molecular events that occur upon substrate binding and serve as a framework for the discovery of new inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Putative alternative spermidine biosynthetic pathway found in *C. jejuni*. The decarboxylation of arginine by arginine decarboxylase (ADC) produces agmatine. Agmatine deiminase/iminohydrolase (ADI) generates N-carbamoylputrescine which is hydrolyzed by N-carbamoylputrescine amidohydrolase (NCP) to putrescine. The addition of aspartate β -semialdehyde to putrescine, catalyzed by carboxyspermidine dehydrogenase (CASDH), yields carboxyspermidine, which is decarboxylated by carboxyspermidine decarboxylase (CASDC) to give spermidine. Adapted from Hanfrey *et al.*¹³

Figure 2.

Structure of CjADI. The overall fold of CjADI is shown (A, helices are colored blue, strands are colored green, and loops are colored yellow) with four of the conserved active site residues (D82, H199, D201, and C315) highlighted in ball and stick representation (green carbon atoms, red oxygen atoms, blue nitrogen atoms and yellow sulfur atom).

Superposition of CjADI (B, shown in blue) and the ADI from *H. pylori* (3HVM, 45% sequence identity, shown in grey, RMSD = 0.886 Å) or the ADI from *A. thaliana* (C, 1VKP, 28% sequence identity, shown in yellow, RMSD = 1.092 Å) shows a very high degree of conservation in the overall structure of the protein. A non-conserved, short helical insert in the *A. thaliana* structure is colored red.

Figure 3.

Structure of the CjADI active site. Crystals of the unliganded C315S mutant of CjADI were soaked in a solution containing 3 mM agmatine to yield a CjADI-agmatine complex structure (A; protein carbon atoms are colored green, agmatine carbon atoms are colored yellow, nitrogen atoms are colored blue and oxygen atoms are colored red). The substrate position and orientation was readily determined from clear difference density in the active site (the electron density shown in A, contoured at 3σ , is from a difference map generated before adding the substrate to the model). A water molecule, presumably the water that hydrolyzes the product at the last stage of the catalytic cycle, was also found in the active site. The catalytically important residues (C/S315, D82, H199 and D201) are all found within hydrogen bonding distance of the guanidinium group of agmatine (B). Several aromatic groups, including W79, W104 and F108 line the binding pocket. The two tryptophan residues, in particular, flank the aliphatic backbone of the substrate.

Figure 4.

Binding pocket of CjADI. This binding pocket of this enzyme is centrally located on the protein (A) and consists of a deep, narrow channel (B, cut away view of the binding pocket of the CjADI-agmatine complex – two of the active site aspartate residues are shown for reference; note that the orientation of the protein in B is rotated 90° about the y-axis with respect to the structure shown in A). A group of polar amino acids (C) surround the entry to the active site and are likely responsible for recruiting the guanadinium group of the substrate into the active site in the appropriate orientation for catalysis.

Figure 5.

Conformational changes upon substrate binding. A structure of the unliganded CjADI (the substrate is shown for reference and is positioned based upon the CjADI-agmatine complex structure) colored by B-factors (low – blue, green, yellow, orange, red – high). Regions around the active site have the highest B-factors. Superposition (B, RMSD = 0.254 Å) of the CjADI-agmatine complex structure (green) onto the unliganded CjADI structure (yellow) shows two specific regions (highlighted with red boxes) where shifts in the secondary structure occur. Closer examination of the active site of the superimposed structures (C; unliganded structure is shown in yellow while the CjADI-agmatine structure is shown in green; atom color scheme same as in Fig. 3 except that agmatine has black carbon atoms in this case) reveals some subtle changes in the positions of the two tryptophan residues as well as a large reorganization of a loop that swings several of the active site residues into binding distance to the substrate. Note that C315 is modeled in two alternate conformations in the unliganded structure to best fit the electron density.

Figure 6.

Kinetics of CjADI. The steady-state kinetics of CjADI-catalyzed deimination of agmatine was measured by quantifying the production of ammonia with a glutamate dehydrogenase-dependent coupled reaction (see Materials and Methods). Despite the structural similarity, CjADI was unable to deiminate arginine.

Figure 7.

Deletion of the ADI gene makes *C. jejuni* more sensitive to aminoglycoside antibiotics. A disk diffusion assay was used to measure the sensitivity of wild-type (WT, 81116, diamonds) and mutant (Mut., C8J_0892, squares) *C. jejuni* to the antibiotics, gentamicin and streptomycin. An increase in the halo size in the mutant indicates a increased susceptibility to the antibiotics. When exogenous spermidine (Spe.) was added to the media, the mutant exhibited a similar level of resistance as the wild-type (Mut. + Spe., circles). The bar shows the mean of the triplicate experiments (duplicate for wild-type), and p-values were calculated using the Student's *t*-test.

Table 1

Data collection and refinement statistics

	CjADI (unliganded)	C315S CjADI-Agmatine
Data Collection		
PDB ID	6B10	6B2W
Beamline	MacCHESS A1	NE-CAT 24-ID-E
Detector	ADSC Q210 CCD	ADSC Q315 CCD
Wavelength (Å)	0.97700	0.97918
Resolution range (Å) ^a	2.09 – 83.24 (2.09 – 2.14)	2.50 – 85.27 (2.50 – 2.60)
Space Group	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁
Unit Cell Dimensions		
a, b, c (Å)	53.4, 80.296, 83.923	54.528, 80.884, 85.626
β (°)	97.357	95.261
Measured reflections	161138	93595
Unique reflections	40360	25689
Mean I/σ	20.43 (3.8)	11.1 (1.8)
Completeness	96.7 (80.8)	99.7 (99.7)
Redundancy	4.0 (3.5)	3.6 (3.0)
R _{merge} (%)	6.1 (21.7)	12.3 (75.7)
CC _{1/2}	0.983 (0.938)	0.982 (0.452)
Data Refinement		
Resolution Range (Å)	2.09 – 83.24 (2.09 – 2.14)	2.50 – 85.27 (2.50 – 2.60)
Total reflections	38375	24503
Test set	1967 (4.9 %)	1170 (4.6%)
R _{work}	0.2023	0.2004
R _{free}	0.2495	0.2648
No. of protein atoms	5192	5512
No. of ligand atoms	22	20
No. of water atoms	292	381
RMSD from ideal		
Bonds (Å)	0.005	0.005
Angles (°)	1.023	0.935
Mean B factor (Å ²)	38.2	31.2
B factor for water	41.2	29.3
B factor for ligands	42.3	41.1
Ramachandran		
Favored (%)	98.3	95.8
Outliers (%)	0.3	0.2
MolProbity Score ^b	1.24 (100)	1.10 (100)

^aNumbers in parentheses correspond to values for the highest resolution shell.

^bValue calculated by MolProbity – value in parentheses corresponds to percentile (100% is best) when compared to a representative set of structures of comparable resolution¹⁹.

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