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Identification and Characterization of a Putative Chemotaxis Protein, CheY, from the Oral Pathogen *Campylobacter rectus*

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Abstract

Campylobacter rectus is an understudied oral bacterium that contributes to periodontitis. Processes that contribute to the disease-causing capabilities of pathogens, such as chemotaxis, are largely unknown in *C. rectus*. The aim of this study was to better understand *C. rectus* chemotaxis, by examining the *C. rectus* genome for the presence of a *cheY* gene. CheY proteins play a part in chemotaxis by acting as two-component response regulators. Significantly, CheY proteins from several pathogens, including the related species *Campylobacter jejuni*, have been shown to contribute to bacterial virulence. Degenerate PCR, RT-PCR, sequence analyses, and structural modeling showed that *C. rectus* encodes a gene (C*r-CheY*) which shares significant homology with previously characterized CheY proteins. Functional studies of a recombinant form of the protein supports a likely role of Cr-CheY in *C. rectus* chemotaxis. *Cr-CheY* is the first CheY characterized from the oral campylobacters.

Keywords

Campylobacter rectus; periodontitis; cheY; chemotaxis

INTRODUCTION

Periodontitis is an infectious disease of the supportive tissue of the teeth (Philstrom et al., 2005). It infects 10% of the population making it a relatively common infectious disease (Teng et al., 2002). Approximately 13% of patients develop severe forms of periodontitis, which, may result in tooth loss and systemic complications including an increased risk of pregnancy complication (Philstrom et al., 2005; Teng et al., 2002).

Campylobacter rectus is a Gram-negative, motile, oral bacterium that has been implicated as a cause of periodontitis (Columbo et al., 2006; Dzink et al., 1985; Lai et al., 1992). Women with periodontitis are seven times more likely to experience preterm labor than their healthy counterparts (Bobetsis et al., 2006; Offenbacher et al., 1996). Serological data has implied

that *C. rectus* plays a role in the preterm labors of mothers with periodontitis (Madianos et al., 2001). Additionally, a pregnant mouse model has shown the association of *C. rectus* with a decreased survival of pups (Offenbacher et al., 2005; Yeo et al., 2005). Although these studies have established *C. rectus* as an agent of periodontitis, the mechanisms of *C. rectus* pathogenesis are not well known.

The genes important to the pathogenesis of *C. rectus* have not been well characterized. A few studies have identified potential virulence factors in *C. rectus*, including toxin genes (*csxA* and *csxB; csxC and csxD*), a potential cell invasion gene (*ciaB*), and a surface array protein (*crsA* gene) thought to play a role in avoiding the host immune system (Braun et al., 1999; LaGier and Threadgill, 2008; Wang et al., 2000; LaGier and Threadgill, 2014). Within the campylobacters, virulence factors have been best characterized from the gastrointestinal pathogen *Campylobacter jejuni* (Young et al., 2007). In *C. jejuni*, chemotaxis is important to establishing disease, by allowing the bacteria to locate an optimal site for infection (Zautner et al., 2012). Among the chemotaxis proteins in *C. jejuni*, CheY is believed to act as a response regulator that interacts with flagellar motor proteins (specifically FliM, when phosphorylated by the signal-transducing histidine kinase CheA) to influence chemotaxis (Marchant et al., 2002). In addition, mutant *C. jejuni* lacking CheY do not efficiently colonize mice, and do not induce campylobacteriosis in ferret models (Yao et al., 1997).

In this study, a *cheY* gene from *C. rectus* was discovered and characterized (*Cr-cheY*) using a combination of bioinformatics, PCR, reverse transcriptase PCR (RT-PCR), and functional complementation experiments in *Escherichia coli*. Interestingly, *C. rectus* appears to be capable of moving within a host from one site to a distant site of infection; specifically, from the subcutaneous tissue to the placenta of pregnant mice (Offenbacher et al., 2005; Yeo et al., 2005). *C. rectus* is also capable of chemotactic behavior *in vitro* (Paster and Gibbons, 1986). Hence, chemotaxis may contribute to the systemic complications of *C. rectus*-related periodontitis. Additional characterization of *C. rectus* chemotaxis, including Cr-CheY, has the potential to identify new ways to disrupt this process.

MATERIALS AND METHODS

Bacterial strains and DNA isolation

Campylobacter curvus (ATCC 33273) and *Campylobacter rectus* strains 314 and 33238 (LaGier and Threadgill, 2008) were grown under standard anaerobic conditions. *C. curvus*, like *C. rectus*, is an oral bacteria associated with periodontitis (Muchach and Tanner, 2000). Bacteria were grown on tryptic soy blood agar with sodium formate (0.3%). Genomic DNA was isolated from bacterial pellets using CTAB (Ausubel et al., 1990). For the complementation studies, an isogenic mutant strain of *E. coli* lacking CheY function (RP5232, Δ*cheY*) and the corresponding wild-type strain (RP437, *WT*) were used (Bourret et al., 1990).

Degenerate PCR

Genomic DNA from *C. rectus* 314 and 33238 were PCR scanned for *cheY* genes using oligonucleotides 5'-GATATGCCTATYATNATGGTWAC-3' (CheY-DF) and 5'-

TTAAWACTTGNGGMGTAAAAGG-3' (CheY-DR). CheY-DF and CheY-DR were designed to amplify the entire *cheY* gene (Wren et al., 1992). Each 50 μl PCR reaction contained 0.5 μM of each primer. The cycling conditions used were as follows: 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute (10 cycles); followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. Amplicons were purified (QIAquick gel extraction kit, Qiagen, USA) and cloned for sequencing into pCR2.1-TOPO (TOPO-TA kit, Invitrogen, USA).

RNA isolation and reverse transcription PCR

RNA was isolated from bacterial pellets using a QIAgen miniprep kit (Qiagen, USA). The RNA was then used for RT-PCR using the Access RT-PCR kit (Promega, USA). Primers CheY-RTF1 (5'-ATCCTAGGTTTATATCG-3') and CheY-RTR1 (5'-TAGGTAAAGCCGAGGTC-3') were used for amplification of *cheY* transcripts.

Recombinant plasmid construction and expression in *E. coli*

The complete open reading frame of *C. rectus cheY* (strain 314, GenBank Accession #EU119866) was cloned (BamHI and HindIII sites) into *E. coli* expression vector pQE80L (Invitrogen, USA) to create pQE80L-Cr-CheY. The engineered plasmid contains the complete *cheY* gene with an in-frame, N-terminus histidine tag. The fidelity of the construct was checked by DNA sequencing. The plasmid pQE80L-Cr-CheY was transformed into *E. coli* RP5232 and RP437. The expression of pQE80L-Cr-CheY was confirmed by SDS-PAGE analysis (Ausubel et al., 1990), after induction of the protein by the addition of 1mM IPTG to overnight cultures of *E. coli* growing in Luria broth; supplemented with 100 μg/mL ampicillin.

Swarming motility assays

E. coli swarming assays were carried out as previously described (Bourret et al., 1990; Li et al., 2006). Briefly, the *WT* (RP5232) and Δ*cheY* (RP437) strains transformed with pQE80L-Cr-CheY were grown overnight at 37°C in LB supplemented with 100 μg/mL ampicillin. A 5 μL sample (10^6 cells) of each culture was place in the center of motility agar plates (Li et al., 2006) supplemented with 100 μg/mL ampicillin and 0.0 or 10.0 μM IPTG. In pQE80L-Cr-CheY, the expression of the recombinant protein is under the control of an IPTG-inducible promoter. The plates were incubated at 30°C. After 24 hours, swarming was assessed by measuring the distance moved (radii measurements) by *E. coli* cells from the original site of inoculation. Three independent experiments were performed and the swarming radii were averaged. Each experiment contained replicate plates for each condition tested. From each plate, three radii measurements were taken.

PCR-based site directed mutagenesis

The QuikChange II site directed mutagenesis kit (Stratagene, USA) was used to change a residue important to the predicted functionality of Cr-CheY. The kit was used according to the manufacturer's protocols, and the change in residue was from a conserved aspartate to an alanine. The PCR template used was pQE80L-Cr-CheY, and CheY-MUTF1 (5' -TGCTTATCACCAACTGGA -3') and CheY-MUTR1 (5' -

GCATGTTCCAGTCGGTGATA-3') were used as primers to introduce the point mutation. The intended change was confirmed by sequencing the resultant plasmid, pQE80L-Cr-CheY-MUT. This plasmid was subjected to motility assays after transformation into *E. coli* RP5232 and RP437.

Sequence analysis and bioinformatics

DNA chromatographs were edited and assembled using Vector NTI software (Invitrogen, USA). Obtained sequences were then used as templates for sequence homology searches using BLAST. Searches were performed using the default parameters for BLASTx (Altschul et al., 1990). Alignments were generated using T-Coffee (Thompson et al., 1994). The resulting alignment was used to generate a WebLogo (Crooks et al., 2004).

I-TASSER was used to predict a tertiary structure for Cr-CheY (Zhang, 2008).

RESULTS

Identification and expression of *C. rectus cheY*

Degenerate PCR was used to amplify a potential *cheY* gene from *C. rectus* genomic DNA. PCR yielded an amplicon of the expected size (366 base pairs, Figure 1A). Amplicons of the correct size were also detected from *C. rectus* 33238 and *Campylobacter curvus* 33273. Cr-CheY from strain 314, which is the most widely studied strain of *C. rectus*, was used in all subsequent experiments. RT-PCR showed the expression of *Cr-cheY* (Figure 1B), with an expected amplicon of 151 base pairs generated from isolated *C. rectus* RNA. DNA sequencing of the degenerate PCR amplicon revealed a *Cr-cheY* ORF of 366 base pairs. The 366 base pair sequence is available at NCBI (#EU119867.1). The open reading frame (ORF) included a start codon (GTG), stop codon (TAA), and translation of the ORF reveals a protein of 121 amino acids, with a predicted molecular weight of 13.7 Kilodaltons.

In silico characterization of Cr-CheY

BLASTx analysis of Cr-CheY revealed a *C. rectus* gene sharing significant sequence identity and similarity to CheY proteins from campylobacters, including *C. jejuni* (NCBI #YP_179249.1; 88% identity, and E-value of 5e-71). A WebLogo (Figure 2, based on a T-Coffee alignment) of Cr-CheY showed homology with CheY proteins, including the presence of conserved residues that contribute to the function of CheY as a response regulator. In the model bacterium *E. coli*, CheY interacts with the flagellar motor switch protein FliM (when phosphorylated by the histidine kinase CheA) to influence chemotaxis (Bourret et al., 1990). Cr-CheY contains a conserved site for phosphorylation (Zautner et al., 2012) by CheA (aspartate, D53 in Cr-CheY; D71 in the WebLogo), and residues involved with binding FliM and CheA (Figure 2). The CheY from the related species *C. jejuni* contains the same conserved sites (Yao et al., 1997); and preliminary BLASTx analysis has identified potential FliM (NCBI #WP_002945945.1) and CheA (WP_002943755.1) homologs from *C. rectus*. The structure of Cr-CheY protein was modeled using the I-TASSER server. As shown in Figure 3, the predicted tertiary structure of Cr-CheY shares significant homology with the known structure of a CheY protein from the related epsilon-proteobacterium *Helicobacter pylori* (protein database #3H1F, http://www.rcsb.org/pdb/

home/home.do); including a conserved arrangement and number of alpha helices and beta strands.

Effect of expressing Cr-CheY on swarming motility of E. coli—The potential function of Cr-CheY was further characterized by functional complementation studies in *E. coli* (**Materials and Methods**). The expression of recombinant Cr-CheY in *E. coli* was confirmed by SDS-PAGE analysis. Following induction of Cr-CheY expression with IPTG, a protein of the expected size (16 Kilodaltons; predicted molecular weight of Cr-CheY plus the weight of the N-terminus histidine tag) was visualized in both *WT* (RP437) and mutant *E. coli* (Δ*cheY*) lacking CheY (RP5232, Figure 4).

The effect of Cr-CheY in *WT* and Δ*cheY E. coli* was assessed by observing changes in swarming motility that requires CheY functionality, and thus requires chemotaxis. While *WT E. coli* display swarming motility, Δ*cheY E. coli* lack swarming motility after 24 hours of growth (Figure 5, **panels A, B, C, D**). Cr-CheY expressed in Δ*cheY E. coli* did not restore swarming, and therefore does not directly replace the function of *E. coli cheY* (Figure 5, **panel F**). However, the expression of Cr-CheY in *WT E. coli* inhibited the swarming ability of *WT* cells (Figure 5, **panel E**). The mean radii swarming distance decreased from 40.0 mm to 6.3 mm (Figure 5, **compare panels C and E**). The inhibition of swarming in *WT E. coli* was not due to differences in growth rates. More specifically, tracking the optical density (600 nanometer readings) of *WT E. coli* carrying Cr-CheY in the presence or absence of 10 μM IPTG showed no difference in growth rate. After 24 hours of incubation, the mean optical densities for samples minus or plus IPTG were 1.81 and 1.80, respectively. As expected, a mutated version of Cr-CheY; in which a key aspartate residue (D53) has been changed to an alanine, restores 100% of swarming motility when expressed in *WT E. coli* (Figure 5, **panel G**). The expression of mutated Cr-CheY in *WT E. coli* was confirmed by SDS-PAGE (data not shown).

DISCUSSION

In this study a *cheY* gene from *C. rectus*, an understudied periodontal pathogen, was identified and characterized. Degenerate PCR, RT-PCR and bioinformatics (Figures 1, 2, and 3) supported the notion that *C. rectus*, which is motile and capable of chemotaxis (Paster and Gibbons, 1986), expresses a gene (Cr-CheY) that shares significant homology with known CheY proteins. *E. coli* strains without CheY lack swarming motility, which is dependent on chemotaxis (Bourett et al., 1990). To begin to characterize the function of Cr-CheY, a recombinant version of the protein was expressed in an *E. coli* strain lacking CheY. Although Cr-CheY did not complement the function of *E. coli* (Figure 5, **panel F**), the expression of Cr-CheY in wild-type *E. coli* inhibited swarming motility (Figure 5, **panel E**). The lack of complementation may be due to insufficient functional similarity. In *E. coli*, chemotaxis is dependent on CheY being phosphorylated by a histidine kinase, CheA (Bourret et al., 1990). The mechanism by which Cr-CheY inhibits *E. coli* swarming is unknown. However, the same effect has been observed in *E. coli* complementation experiments using CheY proteins from *Leptospira interrogans* and *Helicobacter pylori* (Li et al., 2006; Foynes et al., 2000). Interestingly, a mutated version of Cr-CheY expressed in wild-type *E. coli*, which lacks an aspartate residue that is phosphorylated during *E. coli*

chemotaxis, does not inhibit swarming (Figure 5, **panel G**). This observation supports speculation that Cr-CheY expressed in wild-type *E. coli* competes with *E. coli* CheY for phosphates from CheA; and thus inhibits swarming (Foynes et al., 2000; Pittman et al., 2001). Overall, the ability of Cr-CheY to inhibit swarming of *E. coli* suggests that Cr-CheY likely plays a role in *C. rectus* chemotaxis.

Chemotaxis, or directed movement in response to chemical stimulants, has been shown to play a role in the disease-causing capabilities of many bacterial pathogens including *C. jejuni*, *Helicobacter pylori*, *Vibrio cholerae*, and *Listeria monocytogenes* (Yao et al., 1997; Foynes et al., 2000; Butler and Camili 2004; Dons et al., 2004). The protein network in model bacteria that function to direct chemotaxis includes methyl-accepting chemoreceptors (MCPs) that sense changes in extracellular chemical stimulant levels, and cytoplasmic proteins (e.g., cheY, cheA, and cheW) which transmit signals from the MCPs to the flagellum via a series of phosphotransfers (Baker et al., 2006). Although this study focused on CheY, preliminary analysis of the draft *C. rectus* genome suggests that *C. rectus* contains several genes, including MCPs and phosphotransfer proteins, known to play a role in chemotaxis (https://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=KeggMap&page=keggMap&map_id=map02030&gene_oid=644263128&myimg=0). In addition, this study is the first effort to characterize a *cheY* gene from any of the oral campylobacters.

In the related pathogen *C. jejuni*, cells lacking CheY do not swarm, colonize mice, and fail to induce disease in ferrets (Yao et al., 1997). *C. rectus* appears to be capable of moving within a host from one site to a distant site of infection; from the subcutaneous tissue to the placenta of pregnant mice (Offenbacher et al., 2005; Yeo et al., 2005). *C. rectus* is also capable of chemotaxis *in vitro* (Paster and Gibbons, 1986). Most significantly, *C. rectus* is attracted to a potential energy source, formate (Rams et al., 1993), which is also found in periodontal pockets (Paster and Gibbons, 1986). Hence, chemotaxis may contribute to both the systemic complications of *C. rectus*-related periodontitis and assist the bacterium in colonizing subgingival pockets during the development of periodontal disease. Additional studies of *C. rectus* chemotaxis have the potential to discover new approaches for disrupting this process.

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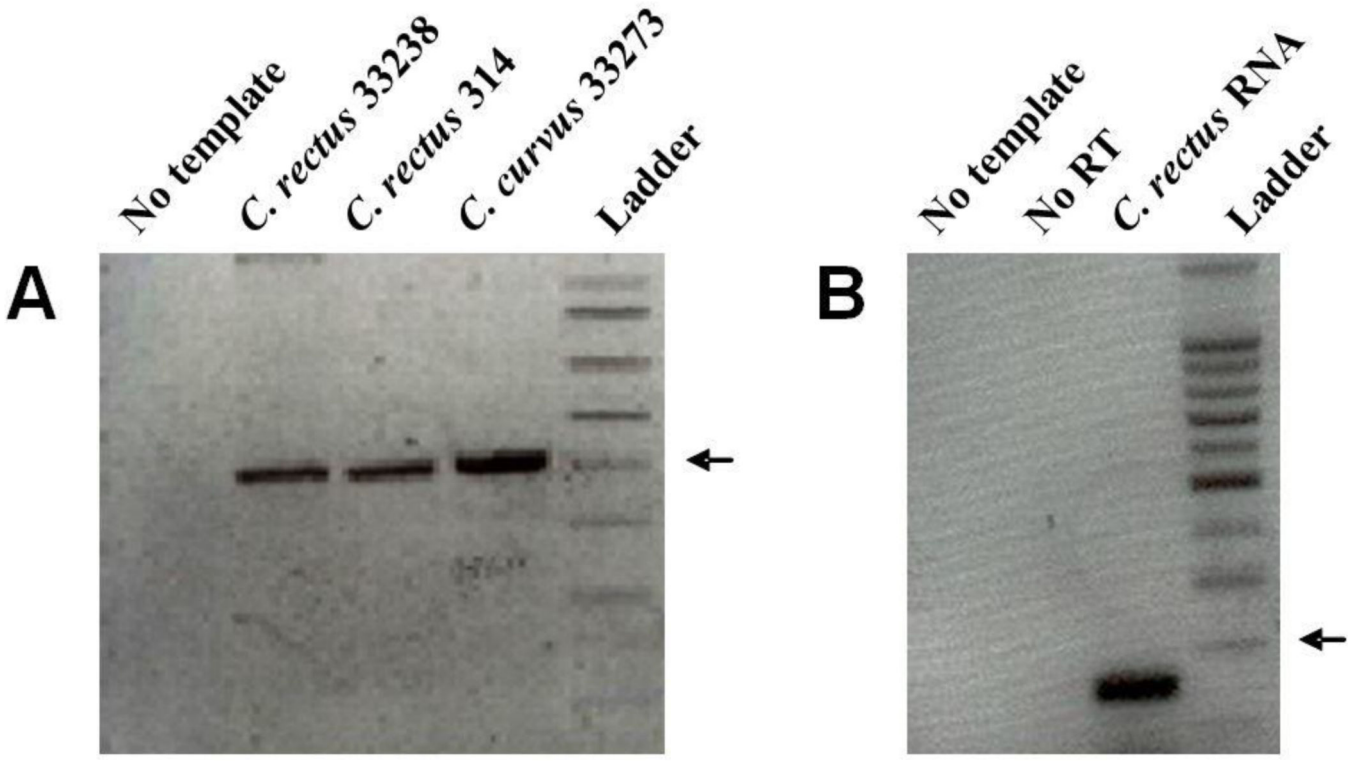

Figure 1.

A. Agarose gel of *C. rectus* or *Campylobacter curvus* DNA amplified by degenerate PCR. An arrow marks 400 base pairs. **B.** Agarose gel of *C. rectus strain* 314 cDNA amplified with RT-PCR. No template = No template control, No RT = No Reverse-Transcriptase control. An arrow marks 200 base pairs.

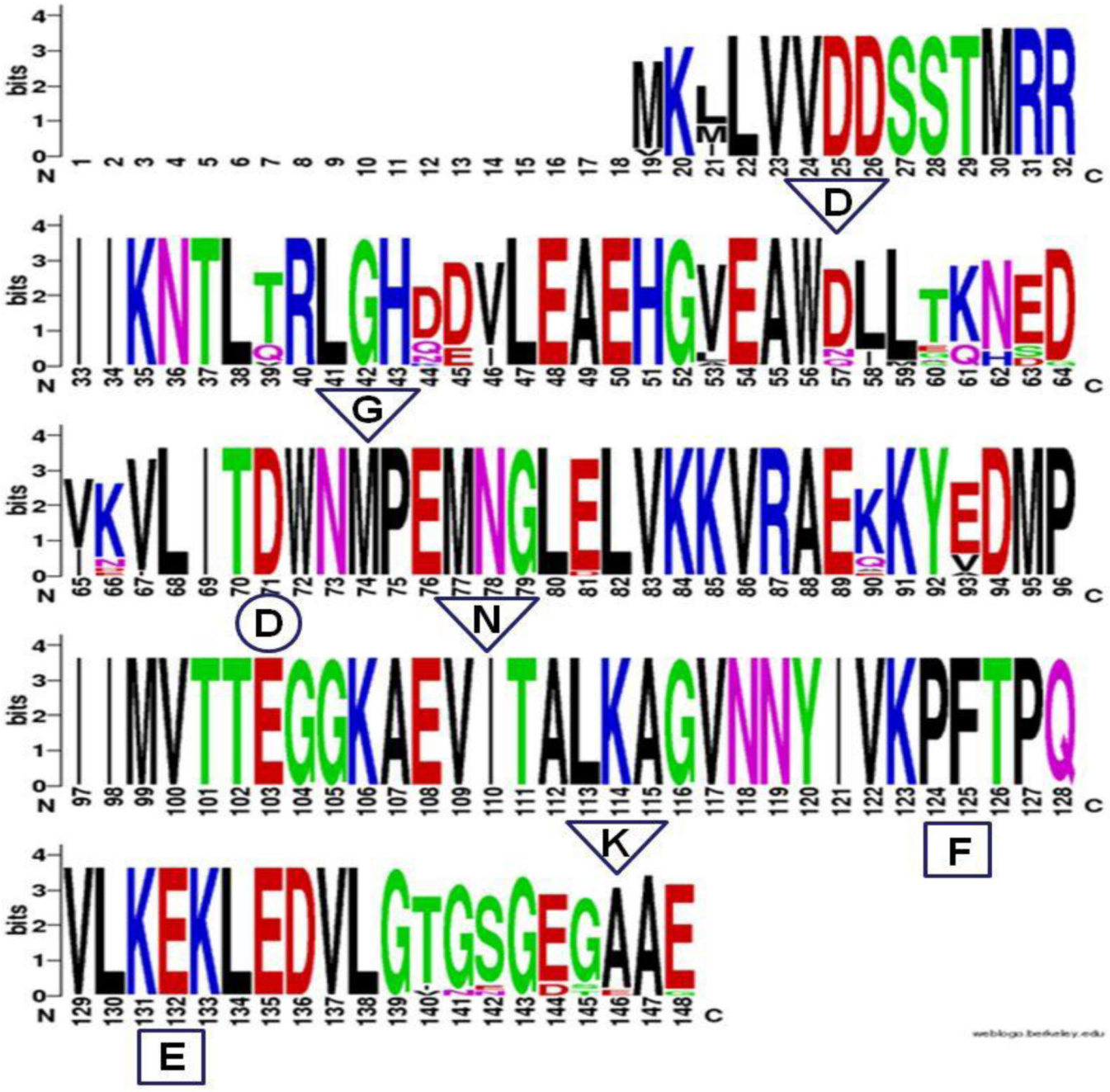

Figure 2.

WebLogo of Cr-CheY and related CheY proteins. The identity and position of conserved amino acids within Cr-CheY are indicated. A score of 4.0 bits indicates 100% conservation of the indicated amino acid among the 24 CheY proteins originally aligned with T-Coffee (**Materials and Methods**). The circled aspartate (D) is phosphorylated by CheA in related CheY proteins. The residues surrounded by rectangles are involved with binding FliM. Residues involved with binding CheA are surrounded by triangles.

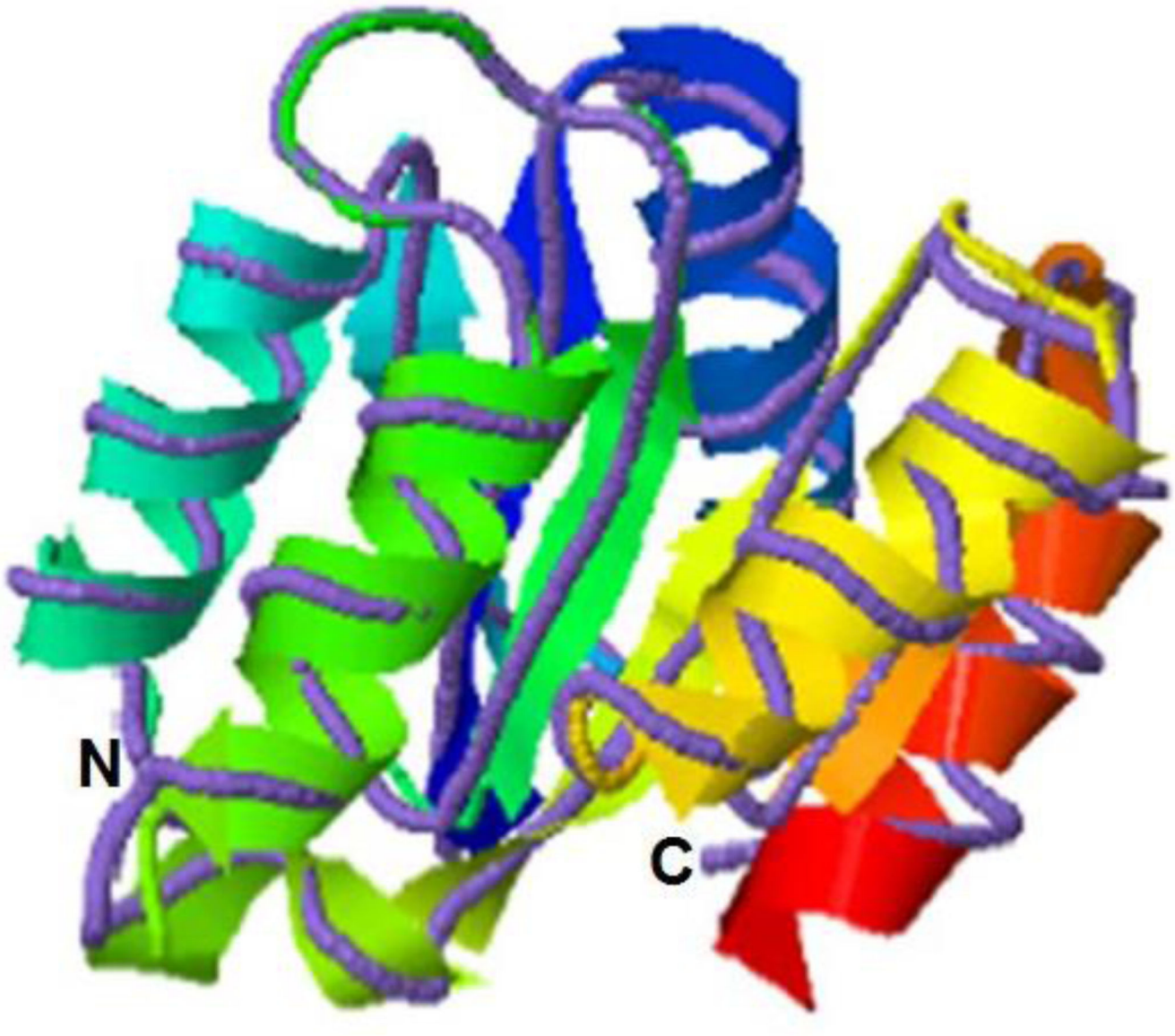

Figure 3.

Model of Cr-CheY structure. The predicted tertiary structure of Cr-CheY (ribbon diagram) shares conserved structural features with the solved structure of a CheY from *Helicobacter pylori* (purple line, protein database #3H1F). The structural similarities are significant according to I-TASSER, with a TM-score of 0.939 (Zhang, 2008). A TM-score of greater than 0.5 is significant. The N and C-termini are indicated.

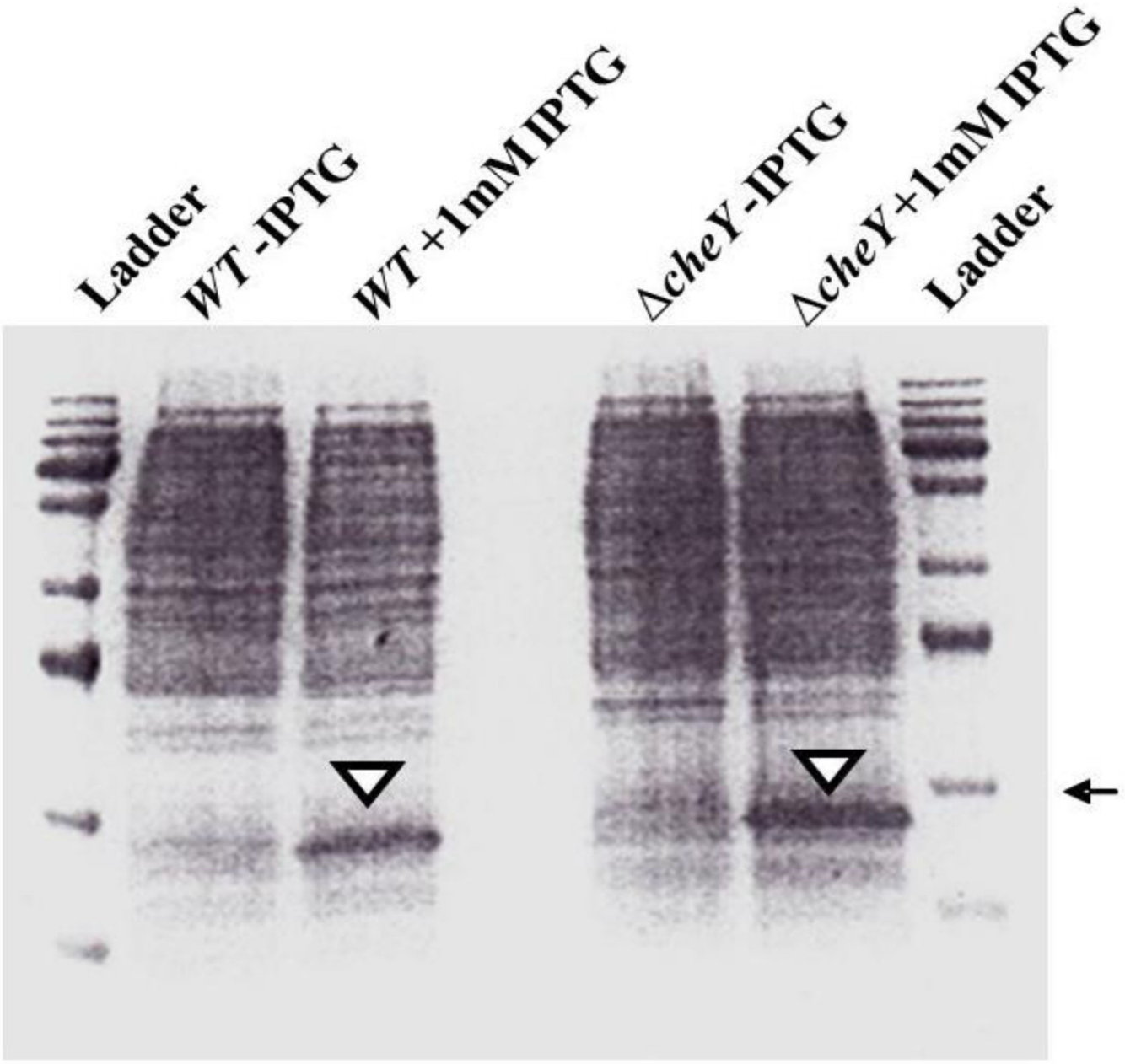

Figure 4.

Coomassie-stained SDS-PAGE gel (15%) showing expression of recombinant Cr-CheY (from *C. rectus*) in *E. coli*. Equal amounts of cell lysate were loaded per well. The arrow indicates 17 Kilodaltons and the arrowheads denote IPTG-induced Cr-CheY at the expected molecular weight (16 Kilodaltons). Ladder = EZ-Run protein standard (Fisher Scientific, USA); *WT* = wild type *E. coli*; Δ*cheY* = isogenic mutant *E. coli*.

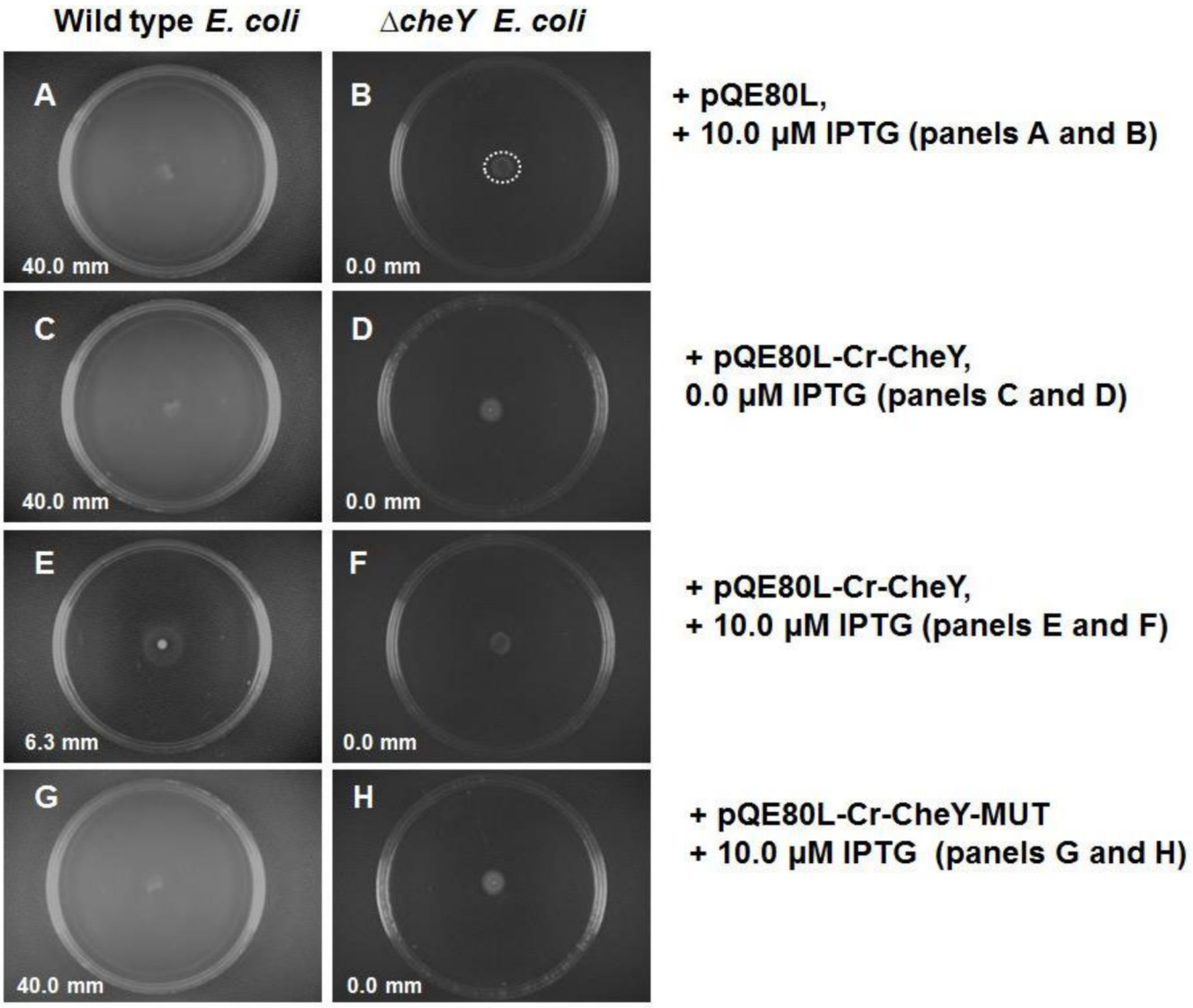

Figure 5.

Swarming motility of *WT* and Δ*cheY E. coli* following IPTG-induced expression of Cr-CheY. Panels A-H show representative swarming motility agar plates. Panels denoted as +pQE80L (A and B) are *E. coli* transformed with plasmid alone (lacking the *cr-cheY* gene from *C. rectus*) and exposed to 10.0 μM IPTG. The number presented in each panel is the mean of swarming radii collected from three independent experiments. Growth at the original inoculation site, as exemplified by the dotted circle in panel B is not considered swarming motility (Bourret et al., 1990; Li et al., 2006). In panels A, C, and G, the entire plate surface is coated by swarming *E. coli*.