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Aspartate chemosensory receptor signalling in *Campylobacter jejuni*.

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Keywords: *Campylobacter jejuni* Tlp, Chemotaxis.

Abbreviations: Tlp, Transducer-like protein; MCP, methyl-accepting chemotactic protein; AD, activation domain; DNA-BD, DNA-binding domain; PAGE, polyacrylamide gel electrophoresis; STD, saturation transfer difference; NMR, nuclear magnetic resonance; IPTG, isopropyl-1-thio- β -D-galactopyranoside; Tar, Aspartate receptor; GST, Glutathione-S-transferase.

Abstract.

The human bacterial pathogen *Campylobacter jejuni* is able to respond to environmental stimuli utilising chemotactic motility. The bacterial senses external molecules via transmembrane sensory proteins called Transducer Like Proteins, TLPs. The specificity of the Tlp1 chemoreceptor (Cj1506c) of *C. jejuni* as the aspartate receptor, CcaA, and its role in chemotaxis signalling pathway were characterised by genetic and biochemical approaches including amino acid and small molecule arrays,

Saturation Transfer Difference NMR spectroscopy, and mutational analysis. Yeast two-hybrid and three-hybrid analysis of protein-protein interactions showed that CcaA chemotactic signal was preferentially passed through CheV, rather than the CheW homologue of the chemotaxis signalling pathway allowing a new model for the *C. jejuni* chemotactic signalling pathway to be postulated.

Campylobacter jejuni is the most common causative agent of human bacterial gastroenteritis worldwide as well as an important part of normal flora in warm blooded animals and particularly birds ^{1, 2}. *Campylobacter* infections in humans are mostly zoonotic in nature and the pathogenicity of this organism is postulated to stem from virulence factors such as chemotaxis, iron acquisition, adherence and surface glycans ³. Similar to other motile pathogenic bacteria, chemotaxis in *C. jejuni* had been implicated to play an important role in infection and disease ⁴⁻⁶. Previous whole cell studies ⁷ demonstrated that *C. jejuni* has the ability to sense its external environment through chemical gradients and have identified a number of chemoattractants and chemorepellents, including amino acids such as L-serine, L-aspartate, L-cysteine and L-glutamate.

The chemotaxis signal transduction pathway is exemplified by the *E. coli* pathway ⁸⁻¹⁰. Periplasmic sensory receptors recognise a chemical ligand and initiate a molecular signal transduction cascade that causes a change in the direction of flagellum rotation and hence the movement of the bacteria towards or away from stimulus. Although chemotaxis pathways of prokaryotes are known to be variable, the fundamental components are conserved and comprise of: a sensory receptor – Histidine Kinase (HK CheA) – a scaffold protein (CheW/CheV) – a Response Regulator (RR CheY). The response regulator ultimately acts on the flagellar motor to switch rotation either clockwise or counter-clockwise.

While the key factors of the chemotaxis signalling pathway can be found in *C. jejuni*, including a CheA histidine kinase, a CheW scaffold protein and a CheY response regulator, deviations from the *E. coli* paradigm exist. Che A protein in *C. jejuni* has an

additional domain similar to the RR CheY and the gene encoding the CheB homologue is unique as it lacks a RR domain usually present in similar proteins of other bacteria. *C. jejuni* also encodes a CheV protein ¹¹ which is homologous to CheV which was originally identified in *Bacillus subtilis* ¹². Che V consists of an N-terminal CheW-like domain fused to a C-terminal response regulator domain.

The chemotactic signal transduction in *C. jejuni* is considered to be initiated by ten putative chemoreceptors and two aerotaxis receptors ¹³. The chemoreceptors are grouped into three classes of methyl-accepting chemotactic proteins, designated as transducer-like proteins (Tlp) in *C. jejuni*. The group A Tlp receptors include Tlp1, 2, 3, 4, 7 and 10 and are proposed to sense ligands external to the cell ¹³. The predicted structures of group A Tlps appear to display classical MCP organisation, similar to that of *E. coli* MCPs, with a periplasmic sensory domain, which is highly variable between different receptors, two transmembrane domains and the C-terminal cytoplasmic signalling domain ¹³. The analysis of the published genome sequences of multiple *C. jejuni* strains demonstrated some diversity in the Group A chemosensory receptor gene content and therefore in how the campylobacters monitor their external environment. Tlp1 was the only receptor universally represented in all sequenced strains with high (98-100%) sequence identity at both the DNA and the amino acid levels ¹⁴. Tlp1 was consequently of primary interest for further analysis.

The expression of the cloned periplasmic sensory domain of the Tlp1 (Tlp1^{peri}) allowed analysis of the ligand binding specificity of this chemoreceptor through the use of the amino acid and small molecule arrays (Day et al., 2009). A protein-ligand

interaction between Tlp1^{peri} and L-aspartate, but no other amino acid, was thus identified and was further confirmed by STD NMR spectroscopy¹⁵.

In order to further analyse the role of Tlp1 in chemotactic signal transduction, an isogenic deletion mutant and complemented isogenic strains were constructed using the *C. jejuni* 11168-O reference strain. Chemotaxis assays (capillary and nutrient depleted) demonstrated that Tlp1 is indeed involved sensing aspartate, as the mutation in the Tlp1 sensory domain coding region resulted in a decreased chemotaxis response towards L-aspartate, while migration of the mutant bacteria towards other known chemoattractants was not affected. The complemented *tlp1*^{-/+} mutant showed restoration of chemotaxis function towards L-aspartate, similar to that of the wild-type. This, in conjunction with the data from amino acid arrays and STD-NMR spectroscopy, conclusively demonstrated that L-aspartate was the only amino acid ligand for Tlp1^{peri} and that *tlp1* encodes the aspartate receptor for *C. jejuni*, *CcaA*¹⁵.

Bioinformatic analysis of the Tlp1 (*CcaA*) sequence revealed an absence of homology of *C. jejuni* Tlp1^{peri} to the previously characterised chemosensory proteins, including that of the aspartate receptor Tar of *E. coli*, one of the best characterised chemosensory receptors to date¹⁶⁻¹⁸. There are marked differences between the Tlp1^{peri} of *C. jejuni* and the Tar receptor of *E. coli* which binds to aspartate and maltose. Maltose, however, exhibits neither chemoattractant nor chemorepellant properties in *C. jejuni*⁷. Future resolution of the Tlp1 3D structure may shed the light on these anomalies.

Fluorescence microscopy and plate-based motility analysis of *tlpI*⁻ isogenic mutants when compared to WT 11168-O and the *tlpI*^{-/+} complemented mutant, indicated a run-biased phenotype for the mutant, as opposed to normal “random walk” which is characterised by alternating tumbling and running of the WT phenotype. It was interesting to note that mutation in the *tlpI* gene affected not only the way the bacteria moved, but also the rates of adherence and invasion in cell culture of CaCo-2 human intestinal cells which were significantly higher for the mutant than for the WT. In contrast the mutant was significantly less able to colonise or maintain colonisation of chickens indicating a possible role for the CcaA in colonisation and possibly pathogenicity of *C. jejuni*.

Aspartate binding to the periplasmic sensory domain of the Tar receptor in *E. coli* modulates the autophosphorylation of bound HK CheA through a MCP-CheA-CheW complex via changes in MCP cytoplasmic signalling domain¹⁷. To investigate whether the CcaA aspartate receptor of *C. jejuni* plays the same role in the chemotaxis pathway, a region homologous to the MCP cytoplasmic signalling domain, identified within the *ccaA* was utilised to demonstrate involvement of CcaA in the receptor signalling complex. The carboxy-terminal residues 501-684 of the CcaA were predicted to contain the cytoplasmic signalling domain of the protein and include the conserved residues involved in the binding of CheW and CheA, based on homology with the *E. coli* Tsr receptor signalling domain. These residues were selected for protein-protein interaction analysis with CheW, CheA, CheY, CheV, CheB proteins and/or their individual domains in Yeast two-hybrid and three-hybrid systems.

The signalling domains of the *E. coli* MCPs interact with the coupling protein CheW to allow formation of MCP-CheW-CheA ternary signalling complexes^{19, 20}. Interestingly, while a weak interaction between *C. jejuni* CcaA signalling domain and CheW was detected, a stronger interaction was identified between the CcaA signalling domain and the CheV protein, specifically with the CheW-like domain of CheV. In *B. subtilis* both CheV and CheW are essential for a complete chemotactic response. CheV could only partially compensate for the deletion of CheW suggesting that CheW and CheV function together to couple CheA to the MCPs in this species²¹. In *C. jejuni*, however, this may not be the case as the three-hybrid analysis and immunoprecipitation assays demonstrated that CcaA has preference for binding CheV rather than CheW protein. When both the CheV and CheW proteins were present, CheV could out-compete CheW for binding to the CcaA1 signalling domain. This suggests that CheV and CheW may share an overlapping binding site on the receptor molecule in the signalling domain, to which CheV binds with greater affinity. It appears likely that *in vivo*, CheV may be predominantly bound to CcaA and there may be minimal, if any, binding of CheW. Differences in the affinity of the other *C. jejuni* Tlps for CheV and CheW were further highlighted in a recent study utilising the high-throughput yeast two-hybrid screens^{22 22} which showed protein-protein interactions between CheW and Tlp4. In addition, interactions were detected between CheV and the Tlps 4, 6 and 8. These findings suggest that the *C. jejuni* Tlps are likely to show variation in their binding capabilities for CheW and CheV and, as a consequence, may signal via alternative pathways controlled by either protein.

CcaA1 was also capable of dimerisation, mediated through interactions in the predicted signalling domain, and is therefore likely to exist as a dimer *in vivo*. This is

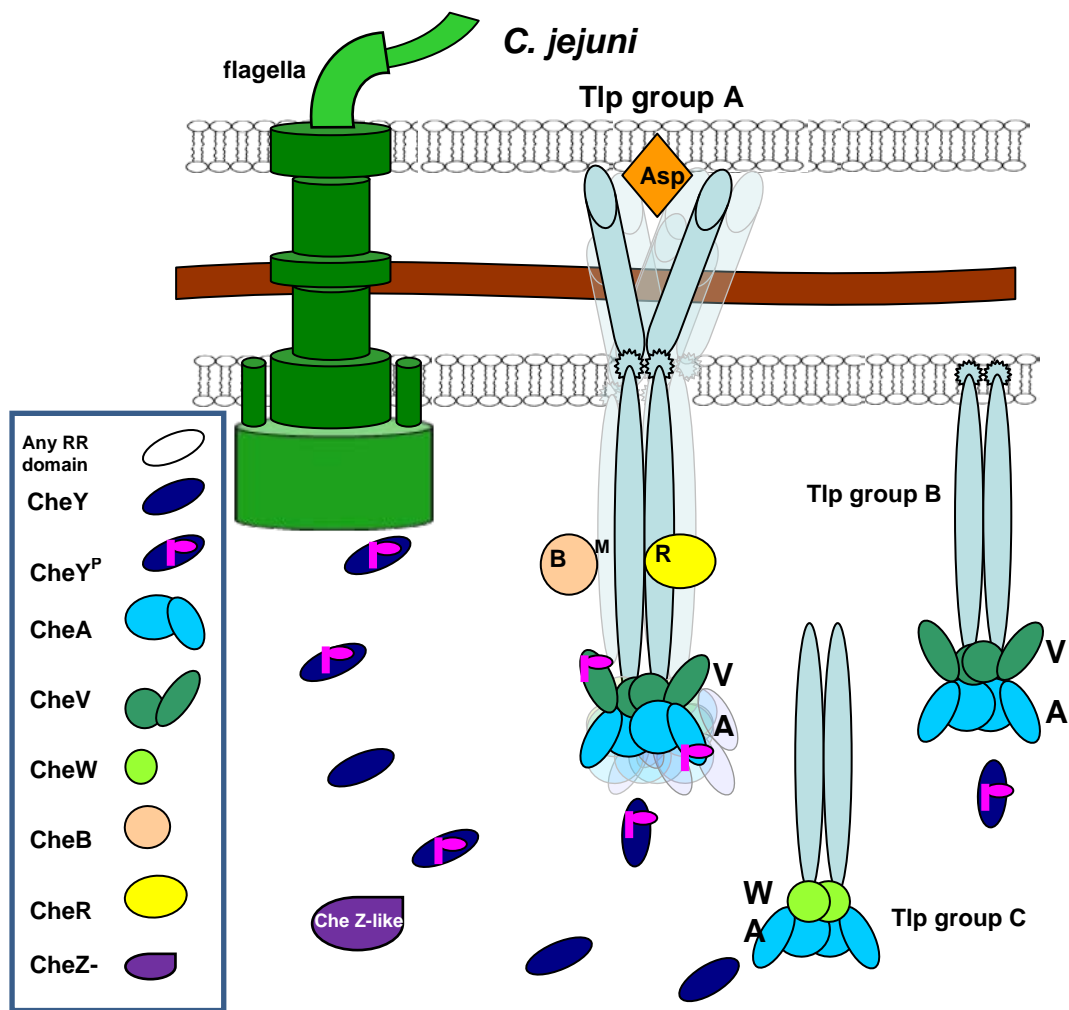
similar to the MCPs of *E. coli*, which exist as homodimers where the C-terminal cytoplasmic domain has been shown to dimerise to form a four-helical bundle structure²³. Moreover, this suggests that CcaA may assemble into higher-order oligomers, such as a trimer-of-dimers or rows-of-dimers, consistent with the *E. coli* paradigm²⁴⁻²⁶. The presence of either the CheV or CheW proteins did not affect the interaction of the Tlp1 signalling domain with itself, suggesting that the region of the signalling domain responsible for dimerisation is distinct from that involved in the binding of CheV or CheW.

The full function of CheV and CheW in the *C. jejuni* chemotaxis pathway is yet to be elucidated, but it is possible to propose a model for *C. jejuni* chemotaxis where signal transduction within the Group A Tlp receptor cluster can be facilitated through either CheV or CheW. In this model, each protein is bound to a specific receptor, such as CcaA but both being present within the heterogeneous cluster of multiple receptors, assuming that the *C. jejuni* receptors cluster in a similar manner to *E. coli* MCPs (Figure 1). In *E. coli*, receptor methylation sites involved in adaptation are not evenly distributed among the receptors and not all are methylated during adaptation. In addition, not all receptors are present in equal numbers in the polar receptor clusters and yet the signal amplification and adaptation processes function equally well for every ligand these receptors recognise^{24, 27}.

It is also tempting to speculate that the Group B and C Tlp proteins may also specifically bind to either CheV or CheW for signal transduction and that every receptor cluster is able to transduce any signal, although at lesser intensity, so long as either CheV or CheW are present in the receptor cluster. Further investigation of the

ligand specificities for the remaining Tlps and their association with CheV and CheW proteins will allow future refining of the chemotaxis model for *C. jejuni*.

Figure 1. Predicted chemotaxis signal transduction model for *C. jejuni*.



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