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***Campylobacter jejuni* ferric-enterobactin receptor CfrA is TonB3 dependent and mediates iron acquisition from structurally different catechol siderophores**

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Abstract

Campylobacter jejuni NCTC11168 does not produce any endogenous siderophores of its own yet requires the CfrA enterobactin transporter for *in vivo* colonization. In addition, the genome of *C. jejuni* NCTC11168 contains three distinct TonB energy transduction systems, named TonB1, TonB2, and TonB3, that have not been tested for their role in siderophore uptake or their functional redundancy. We demonstrate that *C. jejuni* NCTC11168 transports ferric-enterobactin in an energy dependant manner that requires TonB3 for full activity with TonB1 showing partial functional redundancy. Moreover *C. jejuni* NCTC11168 can utilize a wide variety of structurally different catechol siderophores as sole iron sources during growth. This growth is solely dependent on the CfrA enterobactin transporter and highlights the wide range of substrates that this transporter can recognize. TonB3 is also required for growth on most catechol siderophores. Furthermore, either TonB1 or TonB3 is sufficient for growth on hemin or hemoglobin as a sole iron source demonstrating functional redundancy between TonB1 and TonB3. *In vivo* colonization assays with isogenic deletion mutants revealed that both TonB1 and TonB3 are required for chick colonization with TonB2 dispensable in this model. These results further highlight the importance of iron transport for efficient *C. jejuni* colonization.

Introduction

Iron is an essential micronutrient for almost all bacteria [1]. Although iron is one of the most abundant elements on earth, its availability within the host is extremely limited. Iron limitation arises due to its poor solubility under aerobic conditions at physiological pH and due to the presence of the host iron-binding glycoproteins lactoferrin and transferrin [1]. Bacteria thus employ a variety of strategies to acquire the iron sources needed for their growth, including the import of ferrous ions through the high affinity FeoB system [1]. In

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addition, many bacteria produce iron chelators known as siderophores to circumvent the host's attempts to sequester free iron. The prototypical siderophore is the catecholate enterobactin produced by *E. coli* [2]. Enterobactin and other catecholate siderophores such as salmochelin and bacillibactin are powerful iron chelating molecules and are often virulence factors for pathogenic bacteria [3]. Siderophores are secreted into the extracellular medium, where they complex iron and are then imported by highly specific outer membrane proteins (OMPs) in Gram negative bacteria [2–5].

Bacteria that produce multiple siderophores often express separate OMPs dedicated to the transport of each specific siderophore. This is seen in *E. coli* and *Salmonella* sp. that express FepA and IroN for enterobactin and salmochelin transport respectively [6]. However some transporters can recognize and transport multiple siderophores [7]: *Salmonella* IroN can also transport enterobactin and bacillibactin, while *B. subtilis* contains an import pathway that recognizes itoic acid, bacillibactin and enterobactin [8, 9]. The energy required to transport these ligands across the outer membrane in Gram negative bacteria is provided by the TonB-ExbB-ExbD energy transduction system [4, 10, 11]. Through currently undefined mechanisms, the TonB protein transduces the energy from the proton motif force of the inner membrane to the outer membrane receptors to allow for ligand import [4, 12]. While some bacteria such as *E. coli* contain a single TonB complex; many bacteria possess multiple TonB systems [11]. The presence of multiple TonB systems within a single organism implies that each has its own specialized function within the cell. However, given the structural homology of each TonB complex within a single organism, these TonB systems are often able to compensate for each other [10, 13–16].

The food-borne pathogen *Campylobacter jejuni* is a major cause of bacterial enteritis in humans world-wide [17]. Analysis of the *C. jejuni* NCTC11168 genome reveals the presence of multiple OMP iron transporters and the presence of three different TonB-ExbB-ExbD systems [18–21]. *C. jejuni* has been shown to acquire iron through enterobactin, hemin, transferrin/lactoferrin and through ferrous ion import [21–26]. Iron acquisition has also been demonstrated to be an important colonization and virulence determinant in *C. jejuni* [21, 22, 27]. Although *C. jejuni* is unable to synthesize siderophores such as enterobactin, it is able to utilize exogenous enterobactin as an iron source through the action of the CfrA or CfrB transporters [21, 25–27]. Structural characterization of CfrA coupled with homology modelling has suggested that CfrA may interact with enterobactin differently from the *E. coli* FepA receptor and that CfrA may exhibit a broad siderophore recognition range [28].

It is likely that the transport of ferric enterobactin, heme and other iron compounds is TonB dependent in *C. jejuni*, although the role of each specific TonB in iron transport has not yet been experimentally demonstrated. The aim of this study was to investigate the role of TonB1, TonB2, and TonB3 in iron uptake within *C. jejuni*. We report the construction of isogenic deletion mutants into these genes and their functional characterization using siderophore-mediated growth promotion assays. We also assessed the role of each TonB in chick gut colonization. Our work demonstrates that *C. jejuni* is capable of using a wide variety of structurally different catecholate siderophores as sole iron sources. Moreover the majority of these siderophores are transported through the outer membrane protein CfrA and

are dependent on TonB3 for energy transduction. Unlike *E. coli*, *C. jejuni* is able to use both enterobactin and enantioenterobactin as sole iron sources [29]. In addition, while both TonB1 and TonB3 are required for commensal chick colonization, TonB2 is dispensable in this colonization model.

Methods

Bacterial Growth

C. jejuni NCTC11168 was routinely cultured at 37°C in a MACS-VA500 microaerophilic workstation (Don Whitley, West Yorkshire, England) under 83% N₂, 4% H₂, 8% O₂ and 5% CO₂ on Mueller-Hinton (MH) agar plates. The *C. jejuni* strains were tested for motility on 0.4% MH agar plates before performing *in vivo* animal experiments. For the *in vitro* growth assays, the *C. jejuni* wild type and mutant strains were grown in MH broth, MEM α or MEM α supplemented with 40 μ M FeSO₄ at 37°C under microaerophilic conditions. Luria-Bertani (LB) broth and agar plates were used to culture *E. coli* DH5 α at 37°C under aerobic conditions. The strains used in this study are listed in Supplementary Table 1. The construction of the *cfrA* mutant [21] has been previously described and the *chuA* mutant was constructed as described in Ridley *et al.* [23].

Single *tonB* mutant construction

Single deletion mutants of *tonB1*, *tonB2* and *tonB3* were constructed as follows (see Supplemental Figure 1 for the genomic location of the various *tonB* genes). The *tonB1* gene was PCR amplified from *C. jejuni* NCTC11168 chromosomal DNA using primers TonB11 and TonB12 (Supplementary Table 2). The resulting PCR product was purified, digested with BglII and ligated to a BamHI restricted pUC19 to yield pAS16 (Supplementary Table 1). Inverse PCR was performed with the TonB13 and TonB14 primers (containing BamHI restriction sites) to delete a 675 bp region within the *tonB1* gene. The PCR product was purified, digested with BamHI, self-ligated and transformed into *E. coli* DH5 α to yield plasmid pAS20. The kanamycin resistant cassette (*Km*^R) from the pILL600 plasmid was amplified, digested with BamHI and cloned into BamHI digested pAS20 to yield pAS23. The orientation of the *Km*^R cassette was confirmed by DNA sequencing and then transformed into *C. jejuni* NCTC11168 by natural transformation as previously described [30]. Briefly, *C. jejuni* wild-type cells were grown to mid-log-phase in biphasic flasks and 50 μ L aliquots were spotted onto MH plates and allowed to grow at 37°C under microaerophilic conditions for 12 hours. The final construct was added on to the *C. jejuni* lawns and incubated for an additional 12 hours at 37°C under microaerophilic conditions. Thereafter, the lawns were resuspended in 200 μ L MH broth and spread onto MH plates containing kanamycin (30 μ g/ml). Positive colonies were confirmed by PCR amplification of *C. jejuni* mutant chromosomal DNA using primer combinations amplifying the *Km*^R cassette and the respective *tonB* genes. The *tonB2* (AS240) and *tonB3* (AS239) mutants were constructed using the same protocol, except using the chloramphenicol resistant cassette (*Cm*^R) from pRY111 as the selective marker for the *tonB2* mutant. The transformants were selected on MH agar plates containing kanamycin (10 μ g/mL) or chloramphenicol (20 μ g/mL). The use of an alternate antibiotic cassette was to facilitate the construction of double deletion mutants. We also constructed an independent deletion

mutant of *tonB3* using the chloramphenicol resistant cassette (Cm^R) to further confirm the *tonB3* mutant phenotype (AS1135).

Complementation of *tonB3*

Complementation of the *C. jejuni* NCTC11168 *tonB3::cm* mutant strain was constructed as described previously [30, 31]. The *tonB3* gene was amplified from extracted *C. jejuni* genomic DNA using Phusion high fidelity polymerase (Finnzymes) and the *tonB3_SE_comp* and *tonB3_AS_comp* primers (Supplementary Table 2). The amplified gene product was purified using the PureLink PCR Purification Kit (Invitrogen) and subsequently directionally cloned into XhoI (Invitrogen) digested pRRK using the In-fusion Dry-down cloning kit (Clontech) [30]. The pRRK+*tonB3* plasmid was sequenced to confirm the absence of mutations. The *C. jejuni tonB3* mutant strain was then naturally transformed with the final construct and successful transformants were selected for on MH agar plates supplemented with chloramphenicol (20 µg/mL) and kanamycin (30 µg/mL). Positive colonies were confirmed by PCR using the kanamycin cassette specific primer AR56 and ribosomal region specific primers ak233-ak235 (Supplementary Table 2).

Double *tonB* mutant construction

The *C. jejuni* NCTC11168 *tonB1 tonB2* (AS241) mutant was constructed by naturally transforming the *C. jejuni tonB2* mutant (described above) with the final plasmid construct of the *tonB1* mutant. The double *tonB* mutants of *C. jejuni* NCTC11168 *tonB2 tonB3* (AS242) and *tonB1 tonB3* (AS355) were constructed by naturally transforming the *C. jejuni tonB1*, and *tonB2* single mutant strains (described above) with the final plasmid construct for the *tonB3* mutant (Supplementary Table 1). Positive transformants were selected for on kanamycin (30 µg/ml) and chloramphenicol (20 µg/mL) MH agar plates grown under microaerophilic conditions at 37 °C and confirmed by PCR.

Siderophore synthesis and purification

Enterobactin was purified from *E. coli* supernatants as previously described [32]. Salmochelin, linearized enterobactin (Trimer) and (DHB-Ser)₂ (Dimer) were obtained from Prof. Günter Winkelmann (University of Tübingen). Lysine-catecholate was synthesized as previously described [7]. The synthesis of DHB-glycine has been described previously [33]. Synthesis of the siderophore analogs TRENGlyCAM (TGC), TRENSerCAM (TSG), TRENlysCAM (TKC), TRENGluCAM (TEC), TRENbalCAM (TAC), TRENmeCAM(TMC) and TRENbutCAM(TBC) was performed as described elsewhere [34]. The structures of these siderophores and synthetic siderophore analogs are depicted Figure 1.

⁵⁵Fe³⁺-enterobactin uptake assay

Mid-log-phase *C. jejuni* cells grown in iron-limited MEMα medium were pelleted and washed in 10 mM Tris buffer (pH 7.4). The cells were resuspended in uptake buffer to an OD₆₀₀ = 0.6 (~10⁹ bacteria per ml) and kept on ice. The uptake buffer was composed of 5 g/L Na₂HPO₄, 5 g/L KH₂PO₄, 1.18 g/L NH₄Cl, 0.089 g/L Na₂SO₄, 0.042 g/L MgCl₂ hexahydrate and 10 g/L casamino acids. The resuspended cells were allowed to equilibrate

at 37°C for 10 minutes before performing the uptake assay. The $^{55}\text{Fe}^{3+}$ -enterobactin complex used as the iron source was prepared as follows: 10 μL of 10 mM enterobactin was added to 5 μL of 3.6 mM $^{55}\text{FeCl}_3$, followed by the addition of 182.18 μL water and 2.88 μL of 25 mM FeCl_3 . The $^{55}\text{Fe}^{3+}$ -enterobactin complex was incubated at room temperature for 30 minutes followed by the addition of 100 μL of 500 mM sodium phosphate buffer and 700 μL of ddH₂O for a final volume of 1000 μL . This solution was centrifuged at 13000 rpm for 10 minutes and the supernatant was collected. The $^{55}\text{Fe}^{3+}$ -enterobactin solution was diluted by taking 100 μL of the supernatant and mixing with 9.9 mL uptake buffer. This diluted $^{55}\text{Fe}^{3+}$ -enterobactin complex was then filtered twice using a 0.45 μm nitrocellulose filter and used for the uptake assay. The uptake assay was performed using a Millipore filtering assembly (Millipore Corporation, Bedford, MS). One mL of the filtered $^{55}\text{Fe}^{3+}$ -enterobactin complex was added to 10 mL of *C. jejuni* cells (18 nM final $^{55}\text{Fe}^{3+}$ -enterobactin complex concentration). Aliquots of 1 mL were taken at 0, 3, 6, 9 and 12 minutes after the addition of the ferric-enterobactin complex to the cells. The aliquots were passed through a 0.45 μm nitrocellulose filter by vacuum filtration and the filters were washed with 5 mL of 0.1 M citric acid. The filters were then allowed to dry and were subsequently immersed in 5 mL of scintillation cocktail, (Scintiverse, USA), vortexed and counted in a Beckmann LS5000 TD scintillation counter. Final CPM counts were corrected for background. Additional uptake experiments were performed for the wild-type strain as described above except that the protonophore carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) was added to the cells (final concentration 33 μM) three minutes after uptake was initiated. The uptake experiments were repeated at least three times for each condition. Iron uptakes were statistically compared at specific time points using a Student's t test and *P* values below 0.05 were considered significant.

***C. jejuni* growth with various sole sources of iron**

The ability of the *C. jejuni* wild-type and mutant strains to utilize different iron sources was assessed on iron-limited assay plates as described previously [21]. Briefly, the bacterial strains to be tested were grown to mid-log-phase in MH medium, harvested by centrifugation, and resuspended in MH medium to an OD of 1.0 at 600 nm. One mL of this bacterial suspension was added to 25 mL of cooled molten MH agar containing 20 μM desferri-ferrioxamine, poured into petri plates and allowed to solidify. Sterile disc papers containing 10 μL of 10 mM ferric-enterobactin, hemin, hemoglobin and other iron siderophore sources were laid on the surface of the plate and the diameter of the zone of growth promotion was measured after 24 hours of incubation at 37°C under microaerophilic conditions. In addition, 10 mM FeSO_4 was used as a positive control for *C. jejuni* growth. The zone of growth for each mutant was normalized to the corresponding wild type control performed on the same date and reported as a percentage of growth promotion relative to the wild-type strains. The growth promotion assay was repeated at least three times for each iron source and each bacterial strain and the data was statistically analyzed using single-factor analysis of variance (ANOVA). A *P*-value < 0.05 was considered significant. To note, the chloramphenicol deletion construct of *tonB3* (AS11132) displayed the same phenotype with regards to enterobactin usage as the kanamycin deletion construct (AS239).

Chick colonization assays

One day old specific pathogen free chicks (Tyson farms, Arkansas) were checked upon arrival using cloacal swabs to ensure that they were *Campylobacter* free. The chicks were housed at 25°C in a brooder that maintained a temperature in the range of 33–35°C. Commercial chicken starter diet and water were given *ad libitum* to the chicks. For challenge, *C. jejuni* wild-type and *tonB* mutant strains were first grown on motility MH-agar plates under microaerophilic conditions and then transferred to MH biphasic medium. Mid-log-phase bacteria were harvested and resuspended in sterile PBS buffer. An inoculum of 0.25 mL of a bacterial suspension (containing approximately $\sim 10^4$ – 10^5 viable bacteria) was fed orally to each bird. Five birds per group were inoculated separately for the wild-type strain or each mutant. The colonization potential was determined four days post challenge as described previously [21]. Briefly, the ceca were collected following euthanasia, weighed and the contents were homogenized and serially diluted onto selective Karmali agar plates. After incubation at 37°C for 72 hours in a microaerophilic chamber, the bacterial load was determined and expressed as CFU/per gram of ceca. Statistical analysis was done using the non-parametric Mann-Whitney Rank sum test with a $p < 0.05$ considered significant.

Competitive *in vivo* chick colonization assays

The competitive colonization studies were done using five chicks per group. Each bird was inoculated with 0.5 mL of a bacterial suspension containing a 1:1 mixture of wild-type to *tonB* mutant strain. Approximately 10^5 viable bacteria of both wild-type and mutant were present in the mixed inoculums as confirmed by plating serial dilutions of the inoculum on MH agar with and without kanamycin and/or chloramphenicol. The ceca were collected four days post inoculation and processed as described above. The homogenized cecal contents were serially diluted in PBS buffer, and plated on Karmali agar plates and Karmali agar plates supplemented with kanamycin and/or chloramphenicol as required. Colonies were counted after the plates were incubated at 37°C for 72 hours in a microaerophilic chamber. The mutant titer was obtained from the CFU recovered on Karmali agar based plates containing kanamycin and/or chloramphenicol, and the wild type bacterial load was calculated by subtracting the number of mutants from the total number of bacteria recovered on Karmali agar plates without antibiotics. Finally, the *in vivo* competitive index, which corresponds to the ratio of output mutant to wild type bacteria recovered divided by the ratio of input mutant to wild type bacteria inoculated was calculated for each bird. Statistical analysis of the data was performed using a single sample Student *t* test with a $p < 0.05$ considered significant.

Results

Construction of *tonB* single and double mutants

C. jejuni NCTC11168 contains three different *tonB* genes, *tonB1*, *tonB2* and *tonB3*. We therefore set out to determine if each TonB system was required for siderophore transport and chick colonization. Since the TonB systems could overlap in function, we generated specific mutants in each system and double mutants that would render only one TonB system functional. Each *tonB* gene was deleted by allelic exchange with antibiotic resistance cassettes. These mutants were also used to construct the double deletion *tonB* constructs

(*tonB1/B2*, *tonB1/B3*, *tonB2/B3*). Attempts to construct a triple *tonB* mutant lacking all the three known TonB systems and to complement the double *tonB* mutants were not successful due to the lack of the appropriate genetic tools in *C. jejuni*.

Transport of ferric-siderophores is *tonB* specific

We then proceeded to test if the *tonB* mutants were defective in ferric-enterobactin uptake. $^{55}\text{Fe}^{3+}$ -enterobactin uptake assays were performed using the wild-type strain and the various *tonB* mutants. We used cells treated with the protophore cyanide-m-chlorophenylhydrazone (CCCP) to control for radioactivity associated with the cell surface and not actively transported into the cell. As shown in Figure 2, there was a statistically significant difference ($p < 0.05$) in the ferric-enterobactin uptake for all of the single and double *tonB* mutants; with the exception of the *tonB2* mutant. Strains that contained a deletion of *tonB1* in the absence of an additional deletion in *tonB3* (ie *tonB1*, *tonB1/B2*) were slightly (but still significantly) affected in enterobactin uptake. However, all the strains that contained a deletion of *tonB3* (ie *tonB3*, *tonB1/B3*, *tonB2/B3*) were completely abrogated in enterobactin uptake and mimicked the +CCCP phenotype.

C. jejuni can scavenge iron from wide variety of siderophores

We performed growth promotion assays on a wide variety of different catecholate siderophores (Figure 3), as well as hemin and haemoglobin, to determine *C. jejuni*'s ability to use these chelators as sole iron sources. The *tonB* mutants were also assayed for growth using the same iron sources. Previous work has established that CfrA is required for enterobactin growth and ChuA is required for hemin growth in *C. jejuni* [21, 23]. Isogenic deletion mutants of these two transporters were included as negative controls. Siderophore mediated growth promotions of the mutants were normalized to mean value of those from the wild-type strain for each specific iron source. The results were subsequently clustered according to their siderophore utilization profile (Figure 3). The mutants segregated into two main clusters based on their iron source usage, with *cfrA*, *tonB3* and *tonB3* double knockouts in one cluster and *chuA*, *tonB1*, *tonB2*, and *tonB1/B2* in the other. The first cluster is characterized by the inability of the mutants to effectively use enterobactin (and related derivatives), the TREN CAM derivatives and Lys-CAM as iron sources. The *tonB3* mutants (single or double) show no growth on any of these compounds and *cfrA* showed limited growth on Lys-CAM and the TREN CAM derivatives. This indicates that CfrA is the sole transporter for enterobactin and related siderophores, although another transporter(s) is able to partially compensate for *cfrA* deletion with regards to TREN CAM/Lys-CAM siderophore usage. TonB3 is absolutely required for both enterobactin and TREN CAM/Lys-CAM related siderophore utilization. Interestingly, TonB3 also contributes to heme/hemin transport. All *tonB3* mutants were affected for heme/hemin utilization within *C. jejuni* with the *tonB1/B3* double mutant completely inhibited in its ability to use heme/hemin as an iron source. The second cluster is composed of *chuA* and *tonB1*, *tonB2*, and *tonB1/B2*. The ChuA transporter was only required for hemoglobin/hemin utilization and *tonB2* displayed minor defects in the utilization of DHB-Gly, TREN gluCAM (TGC), enterobactin and dimer (DHB-Ser)₂. The *tonB1* mutant showed a minor defect in utilization of enterobactin and salmochelin. Since the *tonB3* mutant displayed the most apparent phenotype, we complemented the *tonB3* mutation by inserting a functional copy of *tonB3*

into the deletion mutant's genome. The complemented *tonB3+tonB3* exhibited fully restored ability to use enterobactin as a sole iron source. This result demonstrates *tonB3*'s specific role in enterobactin transport.

The *tonB* mutants are significantly affected in their ability to colonize chicks

The ability of the *tonB* mutants to colonize the gastrointestinal tract was tested in the commensal (chick) animal model for *C. jejuni*. Firstly, the colonization ability of the *tonB* mutants and the wild-type strain were assessed by orally challenging five birds per group separately with approximately 10^4 viable bacteria. Four days post inoculation, the birds were sacrificed, the ceca were recovered and the cecal contents checked for the bacterial load. All the *tonB* mutants were found to be significantly attenuated in colonization as compared to the wild-type strain, with the exception of *tonB2* (Figure 4A). These results indicate that *tonB1* and *tonB3* are essential for cecal colonization of *C. jejuni*. The level of colonization indicated that the double mutants were more severely affected than the single mutants, with the *tonB1tonB3* double mutant below the detection limit of 100 CFU/gram ceca. Secondly, the ability of the *tonB* mutants to colonize the chicken ceca was further tested in competition with the wild-type *C. jejuni* NCTC11168 strain. The birds were inoculated with a 1:1 mixture of the wild-type strain and the individual *tonB* mutants. Five birds per group were tested for the colonization potential of the mutant and the wild-type strain. Four days after inoculation, the ceca were recovered and the contents were then plated on selective media to calculate the competitive index for each bird. As shown in Figure 4B, with the exception of the single *tonB2* mutant, all the other single and double *tonB* mutants were outcompeted by the wild-type strain in the gastrointestinal tract of birds ($p < 0.05$). These results corroborated the findings of the chick colonization assays with individual strains. The double mutants were the most severely attenuated followed by the *tonB3* and *tonB1* single mutants. The competitive indices of the mutants were also determined *in vitro*, and found to be less affected than *in vivo* (Figure 4B). Therefore, the inability of the mutants to colonize *in vivo* is indicative of an *in vivo* specific defect.

Discussion

In this study we report the construction and characterization of the TonB energy transduction systems in *C. jejuni* NCTC11168 (Supplemental Table 3). We have previously demonstrated that the enterobactin transporter CfrA is the sole transporter of ferric-enterobactin in *C. jejuni* NCTC11168 and is essential for *in vivo* colonization [21]. Mutants in *tonB1* and *tonB3* showed statistically significant reductions in their ability to transport enterobactin while the *tonB2* mutant was unaffected. While the *tonB1* showed a slight reduction in enterobactin transport, the *tonB3* phenotype was severely affected and mimicked the +CCCP phenotype. This indicates that TonB3 is the primary energy transducer of enterobactin mediated iron transport through CfrA with TonB1 playing a relatively minor role.

C. jejuni cannot produce enterobactin endogenously and presumably scavenges enterobactin produced by the host's intestinal microbiota [21]. Given this opportunistic use of enterobactin by *C. jejuni*, we speculated that CfrA may have evolved a broad substrate

spectrum to better take advantage of multiple endogenously produced siderophores. To investigate this hypothesis we tested the *C. jejuni* wild type, *tonB* mutants, and *cfrA* mutant for their ability to utilize a variety of enterobactin-like iron sources. These included salmochelin (enterobactin glycosylated at rings 1 and 2), enantioenterobactin and enterobactin breakdown products (linearized enterobactin, (DHB-Ser)₂ and DHB-Gly). We also probed the specificity of this transport by utilizing a series of TREN based analogs that differ in charge, arm-length and bulkiness. In addition, we tested the strains for their ability to utilize lysine bis-catecholate (Lys-CAM). Lys-CAM is structurally related to enterobactin and is a tetradentate chelator as compared to enterobactin's hexadentate chelation. The intracellular iron sources, hemin and hemoglobin were also screened for utilization by the *tonB* mutants as previous work in *V. cholerae* found that multiple TonB systems could allow proper heme uptake and bacterial growth [15, 16].

C. jejuni NCTC11168 was able to utilize all of the iron sources tested, with the exception of TBC (TRENbutCAM). It is likely that the bulky butyl groups on the catecholate rings prevented its uptake through outer membrane transporters. Deletion of *cfrA* resulted in complete abrogation of enterobactin utilization and greatly reduced use of TRENCAM derivatives (with the exception of TRENgluCAM). This indicates that there may be another transporter for TRENCAM derivatives. However both CfrA and this putative transporter are both completely dependent on TonB3 for energy transduction as deletion of *tonB3* resulted in no growth with enterobactin/TRENCAM derivatives and Lys-CAM. CfrA is able to accommodate a number of different substitutions in the TRENCAM derivatives that would change bulkiness, denticity, and/or overall charge of the ferric-siderophore complex. Substitutions affecting the length and charge of the TREN arms did not impede siderophore usage, but the addition of butyl groups to the catecholate groups on TBC prevented the use of the siderophore. These results suggest that CfrA is a promiscuous transporter that can transport a wide variety of structurally different catecholate siderophores and this transport is dependent on TonB3 activity. TonB1 was found to be partially redundant with TonB3 for hemoglobin and hemin use as deletion of both *tonBs* was required to abolish heme utilization. Deletion of *tonB1* also resulted in a slight growth defect when enterobactin or salmochelin were the sole iron sources which may be related to the slight decrease in enterobactin uptake seen in the *tonB1* mutant (Figure 2). The *tonB2* deletion mutant showed minor defects in the use of TGC, enterobactin and (DHB-Ser)₂.

Interestingly, *C. jejuni* was able to grow by using enantioenterobactin as an iron source. Previous studies have demonstrated that while *E. coli* FepA can transport enantioenterobactin, *E. coli* cannot use this siderophore analog for growth [29]. This lack of growth is presumably due to the fact that the enterobactin esterase Fes is stereospecific and cannot cleave the tri-D-serine triactone ring present in enantioenterobactin [29]. The bacillibactin esterase BesA displays a similar phenotype in that it can hydrolyze the tri-L-serine triactone rings in enterobactin and bacillibactin, but not the tri-D-serine triactone ring present in enantioenterobactin [29]. Our results indicate that *C. jejuni* does not have these limitations in its siderophore usage and may therefore possess novel pathways for iron release from enterobactin that are not stereospecific.

Recent work by Zeng *et al.* have provided the first experimental evidence for the presence of novel pathways in *C. jejuni* enterobactin usage [27]. This study has proposed that there are two pathways for enterobactin assimilation in *C. jejuni* species. The authors propose that in *C. jejuni* NCTC11168, ferric-enterobactin is taken up through the CfrA OMP and primarily transported to the cytoplasm via the CeuBCDE ABC-transporter system. Once ferric-enterobactin is within the cytosol Zeng *et al.* propose that the ferric ion is reduced to the ferrous form and thus the iron is released from enterobactin without enterobactin cleavage as recently demonstrated in *E. coli* [35]. Given the relative novelty of this enterobactin assimilation pathway, it would be interesting to investigate if the postulated ferric reductase could reduce both enterobactin and enantioenterobactin. In addition, this study also identified a *C. jejuni* enterobactin esterase (Cee) that displayed similar enterobactin cleavage kinetics as *E. coli* Fes and is responsible for *C. jejuni* NCTC11168's ability to use enterobactin as a sole iron source in the absence of a functional CeuBCDE ABC-transporter system [21, 27]. It would also be interesting to test if Cee shows the same stereospecificity as Fes or whether Cee can also cleave enantioenterobactin. Either of these two enterobactin assimilation pathways could be responsible for *C. jejuni* NCTC11168's ability to grow on enantioenterobactin.

The presence of multiple *tonB* genes has been reported for several microbes including *Vibrio cholerae*, *Vibrio anguillarum*, *Vibrio vulnificus*, and *Pseudomonas aeruginosa* [5, 10, 11, 14–16]. Studies in these diverse species have shown that the different TonB systems have both overlapping and unique functions. This can result in distinctive *in vivo* phenotypes when the different TonB systems are deleted. For example, *V. anguillarum* requires only one of its two TonB systems for virulence [14]. However in *V. vulnificus* each of its three TonB systems can compensate for each other and single deletion mutants are unaffected in virulence [36]. *V. vulnificus* requires double or triple *tonB* knockouts before virulence defects are observed [36]. In contrast, the deletion of either *tonB1* or *tonB2* in *V. cholerae* results in virulence defects [16].

Our results show that *C. jejuni* deletion mutants in either TonB1 or TonB3 are significantly impaired for chick gut colonization (Figure 4). TonB3's importance in chick colonization is likely related to its role in siderophore transport, in agreement with the similar *in vivo* phenotype of the *tonB3* and *cfrA* mutants. In addition, the inability of either TonB1 or TonB2 to compensate for TonB3 *in vivo* or *in vitro* highlights the important function that TonB3 plays in *C. jejuni* physiology. The defect in the *tonB1* mutant's chick colonization could be due to the slight decrease in enterobactin uptake seen *in vitro*, however it should be noted that the *tonB1* mutant growth defect was comparatively minor in our growth promotion assays. Thus, the *tonB1* phenotype could also be due to a defect in the uptake of an unidentified nutrient. Classically, TonB systems have been shown to be involved in the transport of iron complexes (e.g. siderophores) or cobalamin (Vitamin B₁₂) [37]. However, recent work has revealed new TonB-dependant substrates such as copper, nickel and various sugars and thus the potential role for TonB1 in chick colonization may extend beyond iron metabolism [37–39]. The *tonB2* mutant displayed no significant defects when colonizing the chick gut indicating that TonB2 is dispensable in this animal model. In addition, the

chuA mutant was not affected for colonization and this indicates that *C. jejuni* does not require hemin/hemoglobin during commensal chick colonization.

Significantly, while most *C. jejuni* strains contain three TonB genes, certain strains only have one TonB system. In particular, *C. jejuni* 81–176 does not have TonB1 or TonB3 and solely encodes for TonB2 [40]. However *C. jejuni* 81–176 is able to use hemin/hemoglobin as a sole iron source [41]. Given that *C. jejuni* NCTC11168 TonB2 cannot support growth on hemin (Figure 3) this suggests that the functionality of different TonB systems may not be necessarily conserved across *C. jejuni* strains and that different *C. jejuni* strains have developed unique functions for their respective TonB systems based on their own specific environmental requirements. Presumably, the sole TonB in *C. jejuni* 81–176 is constrained in its specificity as it is the only energy transducing system available in this strain. In contrast, the presence of multiple TonB systems in *C. jejuni* NCTC11168 has allowed each TonB system to become more specialized in the transporters that they energize. This specialization has progressed such that the TonB2 system in *C. jejuni* NCTC11168 no longer recognizes the same targets as the TonB2 system in *C. jejuni* 81–176.

Conclusions

This work demonstrates the ability of CfrA to transport a wide variety of structurally dissimilar siderophores and siderophore analogs. These findings highlight the high degree of adaptability present in *C. jejuni* strains and this adaptability may contribute to *C. jejuni*'s ability to successfully colonize a wide variety of hosts and ecological niches. This work has also shown that specific TonB systems are absolutely required for the transport of certain ligands (e.g. TonB3 and enterobactin), while other ligand transport can be energized by multiple TonB systems (e.g. TonB1/TonB3 and hemin). Finally, *C. jejuni* NCTC11168 TonB2's functional divergence from *C. jejuni* 81–176 TonB2 highlights the inherent difficulty in translating results from one *C. jejuni* strain to another, as the role of specific proteins may differ based on the unique context of each genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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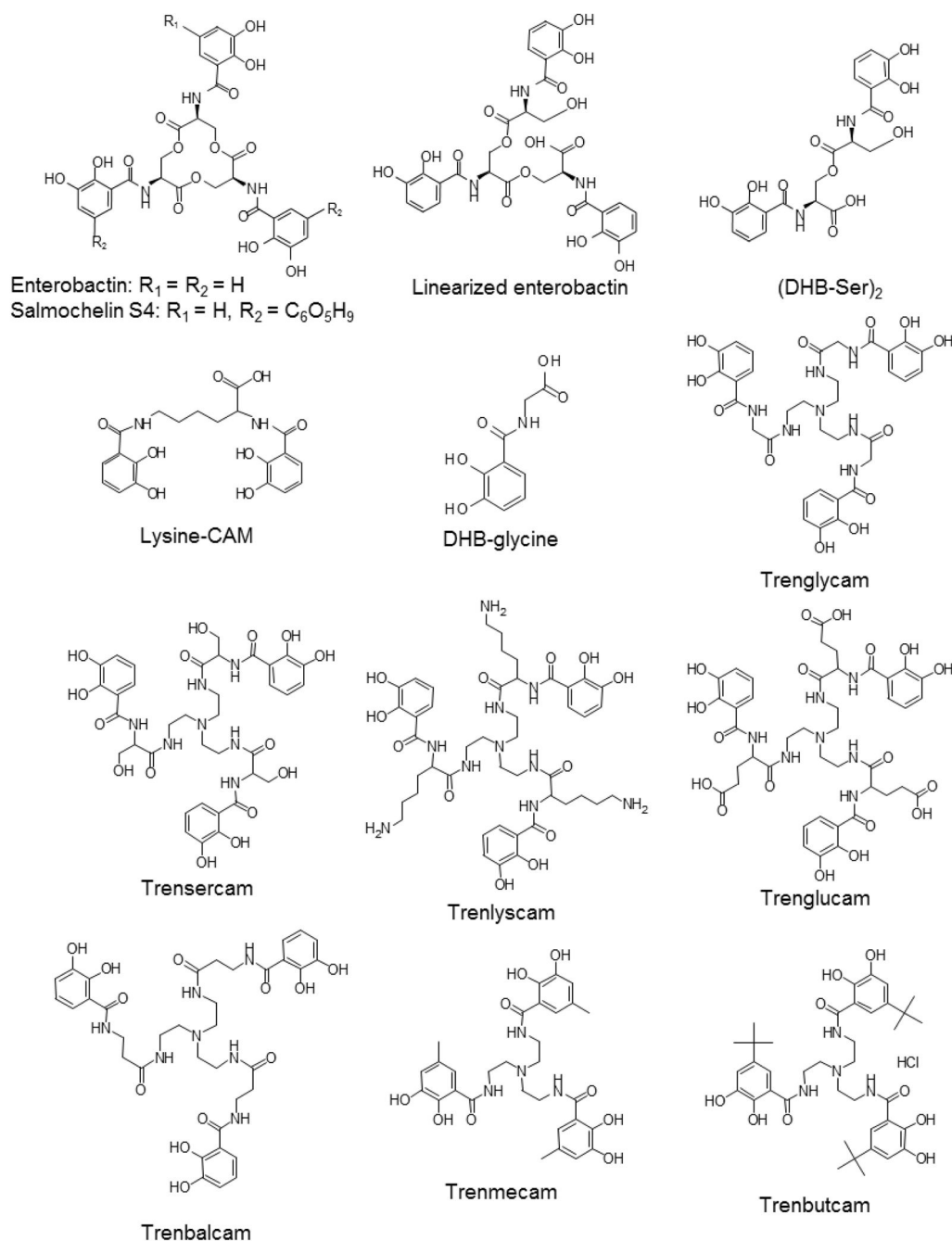


Figure 1. Structures of natural siderophores and synthetic siderophore analogs used in this study
Chemical models of siderophore analogs used in this study. DHB = 2,3-dihydroxybenzoic acid. The TREN (tris(2-aminoethyl)amine) based analogs consist of a TREN backbone, a linker region (gly=glycine, ser = serine, lys = lysine, glu=glutamate, bal= β -alanine) and a CAM (catecholamide) group. TRENmeCAM and TRENbutCAM have additional methyl (me) or butyl (but) groups respectively on the catecholamide ring.

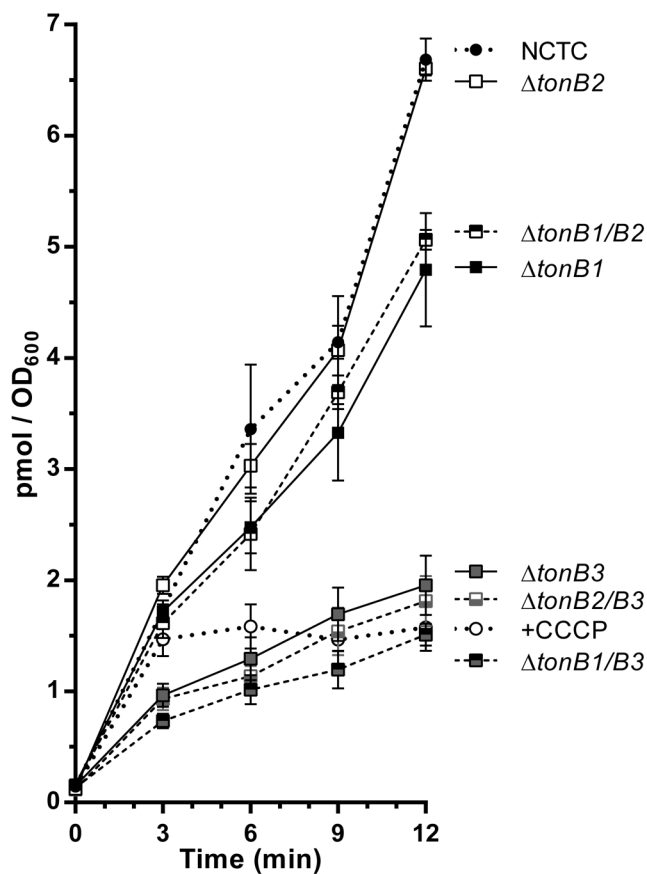


Figure 2. Transport of ferric-enterobactin is TonB specific

Uptake of $^{55}\text{Fe}^{3+}$ -enterobactin by *C. jejuni* NCTC11168 and *tonB* mutant constructs. In the +CCCP sample, NCTC11168 cells were exposed to CCCP 3 min after $^{55}\text{Fe}^{3+}$ -enterobactin addition. Experimental conditions were as described in Materials and Methods.

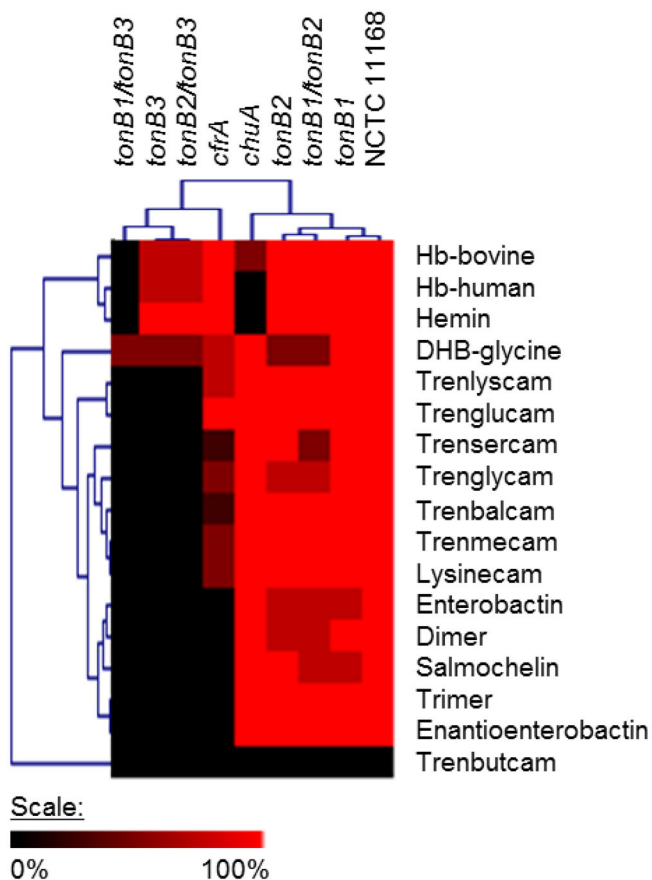


Figure 3. *C. jejuni* can scavenge iron from wide variety of sources

Selected mutant strains were immobilised in MH agar treated with DFO and provided with various iron sources to determine their ability to use each source. The mean growth promotions of each mutant were normalized to those measured for the wild-type strain. The intensity of the red color output for the figure is a function of the relative growth promotion. Black denotes the absence of growth promotion. The results from these experiments were subjected to hierarchical clustering analysis using Pearson’s correlation coefficient.

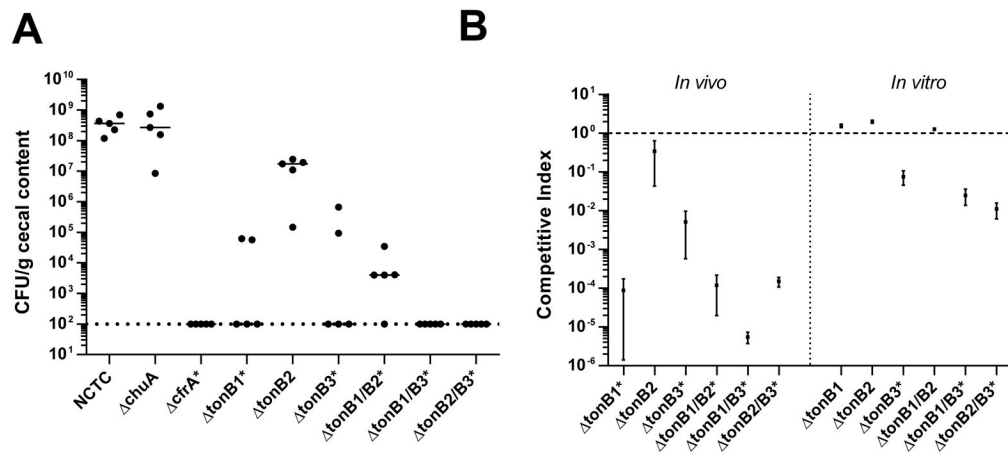


Figure 4. TonB mutants are impaired in their ability to colonize the chick gut

Panel A: Chick colonization studies with NCTC11168 and selected mutants. Five chicks per group were infected with *C. jejuni*. The data points indicate the level of colonization in each chick with the black bar representing the median and the dashed line the detection limit. Strains that showed significant differences as compared to the wildtype ($p < 0.05$) are indicated with an asterisk.

Panel B: Competitive indices of each mutant as compared to the wild type control in competitive association studies with chicks and *in vitro*. Strains that showed significant differences are indicated with an asterisk ($p < 0.05$).