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Specific TonB-ExbB-ExbD energy transduction systems required for ferric enterobactin acquisition in *Campylobacter*

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Abstract

Ferric enterobactin (FeEnt) acquisition plays a critical role in the pathophysiology of *Campylobacter*, the leading bacterial cause of human gastroenteritis in industrialized countries. In *Campylobacter*, the surface-exposed receptor, CfrA or CfrB, functions as a "gatekeeper" for initial binding of FeEnt. Subsequent transport across the outer membrane is energized by TonB-ExbB-ExbD energy transduction systems. Although there are up to three TonB-ExbB-ExbD systems in *Campylobacter*, the cognate components of TonB-ExbB-ExbD for FeEnt acquisition are still largely unknown. In this study, we addressed this issue using complementary molecular approaches including: comparative genomic analysis, random transposon mutagenesis, and site-directed mutagenesis in two representative *C. jejuni* strains, NCTC 11168 and 81-176. We demonstrated that CfrB could interact with either TonB2 or TonB3 for efficient Ent-mediated iron acquisition. However, TonB3 is a dominant player in CfrA-dependent pathway. The ExbB2 and ExbD2 components were essential for both CfrA- and CfrB-dependent FeEnt acquisition. Sequences analysis identified potential TonB boxes in CfrA and CfrB, and the corresponding binding sites in TonB. In conclusion, these findings reveal identities of specific TonB-ExbB-ExbD energy transduction components required for FeEnt acquisition, and provide insights into the complex molecular interactions of FeEnt acquisition systems in *Campylobacter*.

Keywords

Iron uptake; siderophore; molecular mechanism

Introduction

Higher organisms have evolved complicated mechanisms for sequestrating free iron to well below those required for the growth of Gram-negative bacteria (Braun, *et al.*, 1998). To counteract iron-limitation for successful *in vivo* colonization, Gram-negative bacteria have evolved complex and aggressive genetic systems for iron uptake (Braun, *et al.*, 1998, Andrews, *et al.*, 2003, Wandersman & Delepelaire, 2004, Miethke & Marahiel, 2007). For most of the iron uptake systems, iron-regulated outer membrane proteins function as the first line to recognize and bind specific iron complex. Following binding, specific iron complex must be transported through the receptor channel using TonB-ExbB-ExbD energy transduction system, in which TonB is an inner membrane-anchored, periplasm-spanning

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protein while ExbB and ExbD proteins are embedded in inner membrane (Raymond, *et al.*, 2003, Miethke & Marahiel, 2007). The TonB-ExbB-ExbD system transduces the proton motive force energy to the receptor to allow translocation of specific iron source. Therefore, the three-component TonB complex plays an essential role in bacterial iron acquisition.

As the most efficient and common iron scavenging mechanism in Gram-negative bacteria, siderophore-mediated iron acquisition has drawn extensive attentions (Miethke & Marahiel, 2007). In particular, enterobactin (Ent)-mediated iron acquisition has been widely investigated because of the extremely high affinity of Ent to ferric iron (Raymond, *et al.*, 2003) and physiological relevance of Ent utilization for bacterial pathogens (Palyada, *et al.*, 2004, Xu, *et al.*, 2010, Pi, *et al.*, 2012). Recently, we identified and characterized two ferric enterobactin (FeEnt) receptors, CfrA and CfrB in *Campylobacter*, the leading bacterial cause of human gastroenteritis in the United States and industrialized countries (Zeng, *et al.*, 2009, Xu, *et al.*, 2010). Interestingly, analysis of published genome of *C. jejuni* NCTC 11168 (Parkhill, *et al.*, 2000) reveals three sets of TonB-ExbB-ExbD systems, which are all subjected iron regulation (Palyada, *et al.*, 2004). Briefly, the *tonB1* (*Cj0181*) and *tonB2* (*Cj1630*) are organized into the same operon with their corresponding *exbB*/*exbD* genes (*Cj0179*/*Cj0180* and *Cj1628*/*Cj1629*, respectively). The *tonB3* (*Cj0753c*) is an 'orphan' gene with no adjacent *exbB/exbD* genes. There exists a third pair of *exbB*/*exbD* (*Cj0109*/*Cj0110*) in NCTC 11168 genome, which was designated as e*xbB3/exbD3* despite their distant location with the *tonB3.* In this study, we performed genomics and molecular studies to identify the cognate TonB, ExbB, and ExbD components required for CfrA- and CfrB-dependent FeEnt acquisition. The findings from this study provide insights into the molecular interactions and evolution of FeEnt acquisition systems in *Campylobacter*.

Materials and Methods

Bacterial strains, plasmids, and culture condition

The major bacterial strains and plasmids used in this study are listed in Table 1. In general, *C. jejuni* strains were cultivated in Müller-Hinton (MH) broth or on agar at 42°C under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2). To achieve iron-restricted conditions, 20 μM of deferoxamine mesylate (DFO) was added into media. *E. coli* strains were grown routinely in Luria-Bertani (LB) broth with shaking (250 rpm) or on agar at 37 °C overnight. When needed, culture media were supplemented with ampicillin (100 μg/ml), kanamycin (30 μg/ml), chloramphenicol (Cm) (6 μg/ml for *Campylobacter* and 20 μg/ml for *E. coli*), erythromycin (5 μg/ml for *Campylobacter* and 200 μg/ml for *E. coli*) or tetracycline (5 μg/ml for *Campylobacter* and 12.5 μg/ml for *E. coli*).

Construction of *tonB3* mutant

The *tonB3* gene was inactivated by allelic exchange using suicide plasmid as described previously (Hofreuter, *et al.*, 2006, Zeng, *et al.*, 2009). Briefly, the 2,388 bp fragment covering *tonB3* and its adjacent *cfrA* was amplified from NCTC 11168 using primers TonB3F (Table 2) and CfrAR2 (Zeng, *et al.*, 2009), and the PCR product was then cloned into pGEMT-Easy (Promega). The resulting plasmid (pTonB3-CfrA-T) was digested with *BsrG*I and end repaired with T4 DNA polymerase. The tetracycline resistant gene (*tetO*) was PCR amplified from genomic DNA of *C. jejuni* 81-176 using *Pfu* Turbo DNA polymerase (Stratagene) with the primers described in a previous publication (Jeon, *et al.*, 2011); the *tetO* PCR fragment was then ligated to the *BsrG*I-treated pTonB3-CfrA-T, creating the suicide vector pTonB3(tetO). Sequence analysis of the construct indicated that the Tet resistance cassette was inserted into *tonB3* with the same transcriptional direction. This suicide vector was transferred into NCTC 11168 by natural transformation (Wang & Taylor, 1990). The isogenic *tonB3* mutant, named JL832 (Table 1), was selected on MH agar plate

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containing 5 μg/ml of Tet. The inactivation of the *tonB3* in JL832 was confirmed by PCR (data not shown).

Random transposon mutagenesis

we have successfully used *C. jejuni* 81-176 as a host strain for *in vivo* random transposon mutagenesis (Lin, *et al.*, 2009, Hoang, *et al.*, 2011, Hoang, *et al.*, 2012). *C. jejuni* 81-176 cannot utilize FeEnt unless complemented with a periplasmic trilactone esterase Cee (Zeng, *et al.*, 2013). Therefore, we first constructed an 81-176 derivative (JL727, Table 1) that has Cee complementation in chromosome. Briefly, the Cm resistance gene was amplified from plasmid pUOA18 (Wang & Taylor, 1990) with primers Cm_PacI_F and Cm_PacI_R (Table 2) using *Pfu*Ultra® High-Fidelity DNA polymerase (Stratagene). The 0.8-kb PCR product containing a Cm resistance gene was ligated to *Pac*I-digested pCee to obtain vector pCee +Cm in which the Cm resistance gene is inserted between *cee* and its downstream gene *Cj1377c*. Then, the pCee+Cm vector was introduced into NCTC 11168 using natural transformation; the resulting Cm resistant mutant (named JL709, Table 1) has Cm resistance gene between *cee* and *Cj1377c* in the chromosome of NCTC 11168. The genomic DNA from JL709 was further used for natural transformation with *C. jejuni* 81-176 as a host strain, creating strain JL727 (Table 1) in which the *cee* gene together with the Cm resistance cassette was inserted in the chromosome of 81-176. Insertion of the *cee* and the Cm resistance gene was confirmed by PCR; such chromosomal complementation successfully restored 81-176's ability to utilize FeEnt as a sole iron source for growth (data not shown).

The JL727 strain was then subjected to the *in vivo* random transposon mutagenesis; the procedure and screening strategy are detailed in previous publications (Lin, *et al.*, 2009, Hoang, *et al.*, 2011). The mutants with growth defects in MH broth containing kanamycin (50 μg/ml), DFO (20 μM) and Ent (5 μM) were identified. To determine transposon insertion site, genomic DNA extracted from FeEnt-deficient mutants were subjected to direct sequencing as described previously (Lin, *et al.*, 2009, Hoang, *et al.*, 2011).

Chromosomal complementation of CfrB in NCTC 11168

The chromosomal complementation of CfrB in NCTC 11168 was performed using the similar strategy as described previously (Karlyshev & Wren, 2005), in which the target gene is inserted in ribosomal loci. Briefly, the ribosomal region was amplified from NCTC 11168 with primer rrsF and rrlR (Table 2) using GoTaq PCR master mix (Promega). The PCR product was ligated into pGEMT-Easy (Promega), resulting in plasmid pRR. The complete *cfrB* gene together with its promoter was amplified from *C. jejuni* 81-176 with primer pairs of CfrB_XbaI_F and CfrB_XbaI_F (Table 2) using *Pfu*Ultra® High-Fidelity DNA polymerase (Stratagene). Both pRR and the *cfrB* fragment were digested with *Xba*I and *Mfe*I and ligated together, generating pRR-CfrB in which the *cfrB* gene was inserted inside of the ribosomal region. The erythromycin resistant cassette, *erm*, was amplified from genomic DNA of the *cfrA::Erm* mutant of NCTC 11168 (Kindly provided by Dr. Richard D. Haigh, University of Leicester) with primer pairs of Erm_MfeI_F and Erm_MfeI_R using *Pfu*Ultra® High-Fidelity DNA polymerase. The *erm* PCR product was digested with *Mfe*I and was ligated to the pRR-CfrB that was digested with the same restriction enzyme, creating vector pRRE-CfrB in which the *erm* gene is immediately downstream of *cfrB* gene with the same transcriptional direction (Table 1). Approximately 10 μg of the pRRE-CfrB was introduced into JL324, an isogenic *cfrA* mutant of NCTC 11168 (Table 1), by natural transformation. One erythromycin resistant mutant was selected on MH plates containing 5 μg/ml of erythromycin. This mutant, named JL612, has a functional *cfrB* gene inserted in ribosomal region of chromosome, which was confirmed by PCR (data not shown).

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The *tonB2* mutation from a TonB2 mutant (1-3D2, Table 4) was introduced into JL612 by natural transformation, creating mutant JL845 (Table 1). Subsequently, the *tonB3* gene in JL845 was further inactivated by natural transformation using the genomic DNA from JL832, creating mutant JL868 (Table 1)

Complementation *in trans*

The 'orphan' tonB3 gene was amplified from NCTC 11168 with the primer pairs of TonB3F and TonB3R (Table 2) using *Pfu*Ultra DNA polymerase, and then ligated into the *Sma*I-digested shuttle vector pRY107, generating pTonB3 (Table 1). The pTonB3 was further digested with restriction enzymes *Pst*I and *Pac*I, generating a 1.2-kb fragment containing *tonB3;* subsequently, this fragment was ligated to the pCfrA (Zeng, *et al.*, 2009) that has been digested with the same restriction enzymes, creating plasmid pTonB3-CfrA. The plasmid pTonB3-CfrA and pCfrA were conjugatively transferred into *C. jejuni* 81-176 with the help strain JL48, creating complemented constructs JL680 and JL534, respectively (Table 1).

Genomic analysis of TonB-ExbBD

The orthologs shared among finished genomes of *C. jejuni* NCTC 11168 (NC_002163.1) (Parkhill, *et al.*, 2000), 81-176 (NC_008787.1) (Hofreuter, *et al.*, 2006), and *C. coli* RM2228 (AAFL01000001-AAFL01000038) (Fouts, *et al.*, 2005) were compared in Xbase (Chaudhuri, *et al.*, 2008). Other *Campylobacter* genome sequences (finished or draft genomes) were primarily retrieved from CampyDB (http://www.xbase.ac.uk/campydb) or IMG (http://www.hmpdacc-resources.org/cgi-bin/imgm_hmp/main.cgi).

Sequence analysis of CfrA, CfrB, and TonB

The phylogenetical analysis of *Campylobacter* CfrA, CfrB, and TonB together with their homologs in *E. coli* were performed in MEGA 5.0 (Kumar, *et al.*, 2008). The phylogenetic tree was constructed using Neighbor-joining methods. To identify the potential TonB box in CfrA and CfrB and the specific binding sites in TonB for TonB box, multiple-sequence alignment of the sequences from *Campylobacter* as well as *E. coli* were performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Results

Comparative genomics analysis of TonB-ExbB-ExbD systems in *C. jejuni* and *C. coli*

Analysis of several published *C. jejuni* genomes has revealed that *C. jejuni* strains differ in the presence of TonB-ExbB-ExbD systems (Stintzi, *et al.*, 2008, Miller, *et al.*, 2009). In this study, we performed in-depth genomic analysis by taking advantage of the published genome data to date. Table 3 shows the key components of FeEnt acquisition in two *C. jejuni* representative strains (NCTC 11168 and 81-176) and one *C. coli* strain (RM2228). Furthermore, the genomic organization of *exbB-exbD-tonB* loci in different *Campylobacter* strains was compared (Fig. S1).

ExbB3 and ExbD3 may not be cognate components for TonB3 because *tonB3,* in fact, is physically distant from the so-called *exbB3/exbD3* operon. *Cj0111*, the gene immediately downstream of *exbD3* (*Cj0110*) (Fig. S1), encodes a protein with a TonB-2 domain (pfam13103) at C-terminal, suggesting the ExbB3/ExbD3 system may interact with Cj0111. Interestingly, the *exbB3-exbD3-Cj0111-Cj0112-Cj0113* operon resembles the genomic organization of Tol operon (*tolQ-tolR-tolA-tolB-pal*) identified in many other Gram-negative bacteria (Godlewska, *et al.*, 2009) (Fig. S1).

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The *exbB2-exbD2-tonB2* operon is highly conserved and present in all *C. jejuni* genomes. Interestingly, in *C. coli* RM2228, *tonB2* is missing while the complete *exbB2/exbD2* genes are present (Table 3); this unique pattern also was observed in other sequenced *C. coli* strains in IMG (data not shown).

In *C. jejuni* NCTC 11168, a putative iron transporter gene (*Cj0177*) and the transferrin/lactoferrin receptor gene (*Cj0178*) are immediately upstream of the *exbB1-exbD1-tonB1* operon (Fig. S1), suggesting that TonB1 energy transduction system is functionally related to Cj0177 and Cj0178. Cj0177 is a homolog of *E. coli* CjrA transporter (50% aa similarity) that is adjacent to CjrB (a TonB protein) and CjrC (an outer membrane colicin Js receptor) (Smajs & Weinstock, 2001). The TonB1 system was prevalent and highly conserved in *C. coli*. For example, analysis of the sequence data from a recent *Campylobacter* pan-genome project (Lefebure, *et al*., 2010) revealed that the *exbB1-exbD1-tonB1* operon was present in all 42 diverse *C. coli* strains. However, the *exbB1-exbD1-tonB1* operon was missing in 14 out of 59 *C. jejuni* genomes retrieved from IMG.

The TonB2 system is critical for CfrB-dependent FeEnt acquisition in *C. jejuni* 81-176

A library containing 7350 Tn5 mutants was screened for the mutants that failed to grow in the iron-restricted medium supplemented with Ent. Eight mutants were identified, in which five have transposons inserted in *exbB2-exbD2-tonB2* locus (Table 4).

CfrB also utilizes TonB3 for efficient FeEnt acquisition

Despite the critical role of TonB2 system in CfrB-dependent pathway as demonstrated above, TonB3 may also involve CfrB-dependent FeEnt acquisition because inactivation of *tonB3* in *C. coli* VC167, a strain using CfrB as dominant FeEnt receptor, abolished its ability to utilize Ent (Guerry, *et al*., 1997). Thus, we further examined the genetic interaction of CfrB with TonB3 by manipulating *C. jejuni* NCTC 11168. As shown in Fig. 1A, chromosomal complementation of the *cfrA* mutant with *cfrB* completely restored its ability to utilize FeEnt. Inactivation of *tonB2* or *tonB3* alone in this genetically repaired mutant (*cfrA*$^{-}$ *cfrB*$^{+}$) did not affect FeEnt acquisition (Fig. 1A). However, the mutant with mutations in both *tonB2* and *tonB3* genes failed to utilize FeEnt, indicating CfrB also could interact with TonB3 for efficient FeEnt utilization.

TonB3 is essential for CfrA-dependent FeEnt acquisition

Regarding the identity of specific TonB component for CfrA, in *C. jejuni* NCTC 11168, inactivation of TonB3 alone abolished FeEnt utilization while inactivation of TonB2 did not (Fig. 1B). In addition, for *C. jejuni* 81-176 that only contains TonB2 (Table 3), wild-type CfrA gene (pCfrA) failed to rescue it for FeEnt utilization while both *cfrA* and *tonB3* genes (pTonB3-CfrA) restored its ability to utilize FeEnt (Fig. 1B); this evidence further indicated that TonB3 is critical for CfrA-dependent FeEnt acquisition.

ExbB2 and ExbD2 are essential for both CfrA- and CfrB-dependent FeEnt acquisition in *C. jejuni*

As revealed in random transposon mutagenesis experiment, ExbB2 and ExbD2 are essential for CfrB-dependent FeEnt acquisition in *C. jejuni* 81-176 background (Table 4). To further validate this finding, we transferred the *exbB2* and *exbD2* mutations from 81-176 mutants into the CfrB-repaired NCTC 11168 strain JL612 (*cfrA*$^{-}$ *cfrB*$^{+}$). As shown in Fig. 1C, inactivation of ExbB2 or ExbD2 alone completely abolished JL612's ability to utilize FeEnt, further demonstrating the essential role of ExbB2 and ExbD2 in CfrB-dependent pathway.

Regarding the CfrA-dependent pathway that requires TonB3 in wild-type NCTC 11168, single *exbB2* or *exbD2* mutation could completely abolish its ability to utilize FeEnt (Fig.

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1C). This finding indicates that ExbB2 and ExbD2 are also essential for CfrA-dependent FeEnt acquisition even if they are distant from the *tonB3-cfrA* locus; other ExbB/ExbD systems in NCTC 11168, such as ExbB3/ExbD3, cannot compensate the function of ExbB2/ExbD2 for TonB3-mediated FeEnt acquisition.

Predicted TonB box in FeEnt receptors and the corresponding binding sites in TonB

It is interesting that CfrA only interacts with TonB3 while CfrB can interact with both TonB2 and TonB3 for efficient FeEnt acquisition. Since the TonB-dependent FeEnt receptor interacts with TonB through the conserved TonB box that contains 5-7 amino acid residues (Krewulak & Vogel, 2011), we speculate the sequence variation may exist in the TonB box region of CfrA and CfrB. As expected, the predicted TonB box of CfrA is SSIV(/I)S while that of CfrB is DVVVS (Figure 2A); these specific TonB box sequences are highly conserved in the sequenced CfrA and CfrB, respectively (data not shown). Analysis of different TonB proteins indicated that the predicted TonB box binding sites for TonB1, TonB2, and TonB 3 are KFKKYP, QAQFYP, and EALIYP, respectively (Figure 2B); each specific type of TonB from diverse *Campylobacter* strains displays the identical TonB box binding site (data not shown).

The CfrA from *C. jejuni* and *C. coli* form a cluster that is close to the cluster of the CfrB from *C. jejuni* and *C. coli*, indicating they are orthologs of the FepA and IroN from *E. coli* (Figure 2C, left panel). In addition, TonB2 was phylogenetically closer to TonB3 than to TonB1 (Figure 2C, right panel).

Discussion

The presence of multiple TonB-ExbB-ExbD systems in a single organism is not unique to *Campylobacter* although many Gram-negative bacteria, such as *E. coli*, only have one set of TonB-ExbB-ExbD complex. For example, *Helicobacter pylori* 26995 (Schauer, *et al.*, 2007), *Vibrio cholerae* CA401S (Seliger, *et al.*, 2001), and *Pseudomonas aeruginosa* PAO1 (Zhao & Poole, 2000) have two sets of TonB systems. In addition, there are up to eight sets of TonB-ExbB-ExbD paralogs in *Myxococcus xanthus* (Sogaard-Andersen, 2011). Bacteria may acquire multiple TonB systems with different functional specificities during evolution (Seliger, *et al.*, 2001). This study is focused on the identification of cognate TonB system(s) for the two different FeEnt acquisition pathways in *Campylobacter* (Zeng, *et al.*, 2013). We demonstrated that TonB3 plays a dominant role in CfrA-dependent FeEnt acquisition, which is consistent with the finding from a recent report (Naikare, *et al.*, 2013). However, in CfrB-dependent pathway, TonB2 and TonB3 are interchangeable for efficient FeEnt acquisition.

Interestingly, although CfrA and CfrB display different specificity for TonB component, both the CfrA- and CfrB-dependent FeEnt acquisition pathways use the same ExbB/ExbD complex (ExbB2/ExbD2). Our data here suggest the *Campylobacter* ExbB2/ExbD2 is orthologs of the ExbB/ExbD complex in *E. coli* in terms of FeEnt acquisition. The pivot role of ExbB2/ExbD2 was also reflected by the presence of *exbB2/exbD2* in all *Campylobacter* genomes. It has been proposed that ExbD interacts with TonB through the periplasmic domain (Pramanik, *et al.*, 2011, Ollis & Postle, 2012). Thus, future structural and sequence analyses of ExbD2, TonB2 and TonB3 are needed to reveal molecular interaction between ExbD and TonB.

As expected, ExbB3/ExbD3 is not associated with TonB3 for FeEnt acquisition. Consistent with this finding, previous microarray study (Palyada, *et al.*, 2004) has suggested that the ExbB3/ExbD3 complex is not involved in iron acquisition because ExbB3/ExbD3 genes were up-regulated in response to iron repletion rather than iron depletion. The *exbB3, exbD3*, and their downstream genes display similar genomic organization to the *tolQ-tolR*-

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tolA-tolB-pal operon (Fig. S1) that is important in the pathophysiology of other Gram-negative bacteria (Godlewska, *et al.*, 2009). This observation warrants further functional characterization of the ExbB3/ExbD3 in *Campylobacter*.

Current structural model for the interaction between TonB and TonB-dependent receptor is the formation of the β-sheet containing mixed β-strands from TonB and TonB-dependent receptor (also called TonB box) (Shultis, *et al.*, 2006). In this study, we observed that CfrA and CfrB displayed different TonB box sequence with major difference in the first two aa residues. The first two residues of the CfrA TonB box (SS) contain uncharged polar side chain. However, the first two residues of the CfrB TonB box (DV) contain acidic side chain and nonpolar side chain, respectively. Thus, the first two aa residues in the TonB box of CfrA or CfrB may determine its specificity for the cognate TonB component. This hypothesis needs to be examined in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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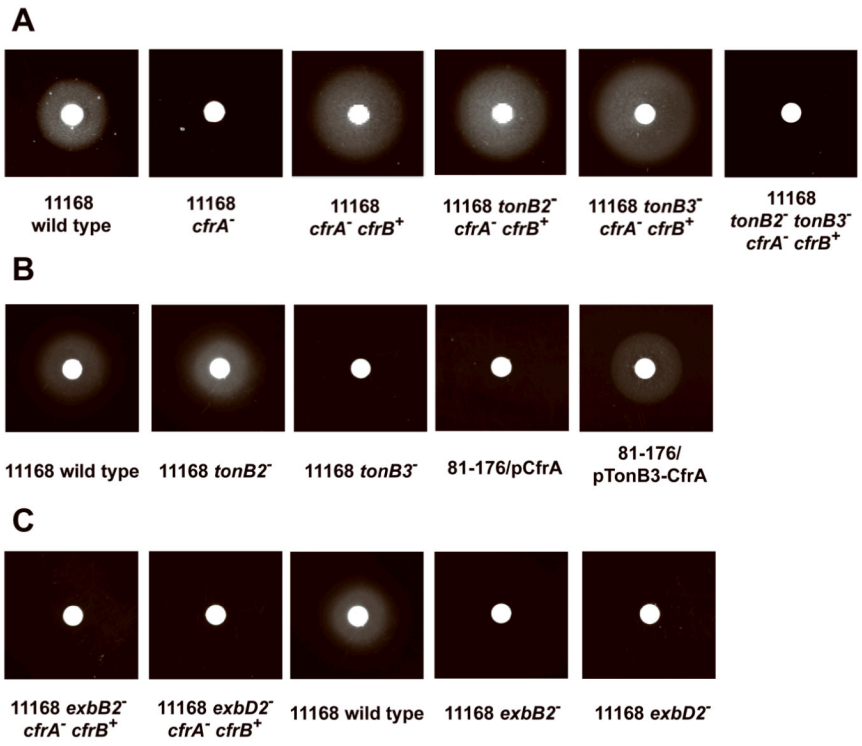

Figure 1.

Identification of specific TonB-ExbB-ExbD components required for CfrA- and CfrB-dependent FeEnt acquisition in *C. jejuni*. Standard Ent growth promotion assay was performed for wild-type strain and its mutant derivatives. (A) TonB3 could interact with CfrB for efficient FeEnt acquisition in *C. jejuni* NCTC 11168. (B) TonB3 is essential for CfrA-dependent FeEnt acquisition. (C) ExbB2 and ExbD2 are essential for FeEnt acquisition in *Campylobacter*.

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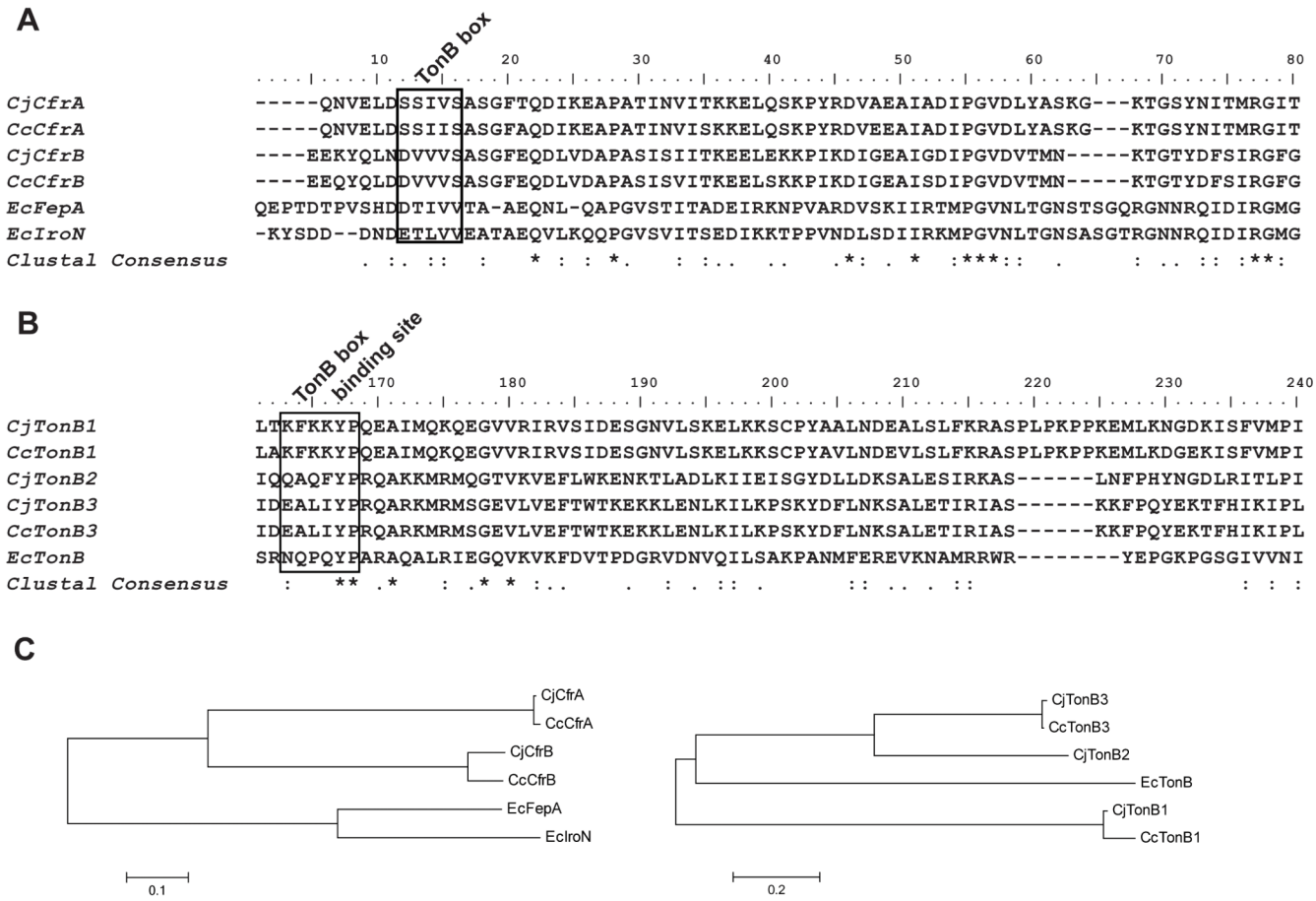

Figure 2.

Comparative analyses of FeEnt receptors and TonB components in *Campylobacter* and *E. coli*. (A) Multiple sequence alignment of FeEnt receptors from *Campylobacter* and *E. coli*. The sequences were aligned with Clustal W2. The region of aa 1-80 was displayed. Putative TonB box region was highlighted in the rectangle boxes. Identical amino acids are marked by an asterisk (*), and conserved and semi-conserved substitutions, substitutions are marked by colon (:) and a single dot (•), respectively. (B) Multiple sequence alignment of identified TonB components in *Campylobacter* and *E. coli*. The region of aa 161-240 was displayed. One putative TonB box binding site was highlighted in the rectangle box. (C) Phylogenetic analyses of FeEnt receptors and TonB components from *Campylobacter* and *E. coli*. Left panel, phylogenetic analysis of *C. jejuni* FeEnt receptors (CjCfrA and CjCfrB), *C. coli* FeEnt receptors (CcCfrA and CcCfrB), and *E. coli* FepA and IroN. Right panel, phylogenetic analysis of *C. jejuni* TonB (CjTonB1, CjTonB2, and CjTonB3), *C. coli* TonB (CcTonB1 and CcTonB3), and *E. coli* TonB (EcTonB).

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Table 1

Key bacterial plasmids and strains used in this study

Plasmids or strains	Description	Source or Reference
Plasmids		
pGEMT-Easy	PCR cloning vector, Amp^r	Promega
pCee	T-easy vector derivative containing *Cj1376* and partial *Cj1377c*	(Zeng, *et al.*, 2013)
pCee+Cm	pCee derivative with *cat* cassette inserted at *PacI* site between *cee* and *cj1377c*	This study
pRR	T-easy vector derivative containing *C.jejuni* NCTC 11168 ribosomal fragment	This study
pRR-CfrB	pRR derivative in which *cfrB* gene was inserted inside ribosomal region	This study
pRRE-CfrB	The erythromycin resistant cassette was attached at the end of *cfrB* in pRR-CfrB	This study
pRY107	*E. coli-Campylobacter* shuttle vector, kanamycin resistant (Km^r)	(Yao, *et al.*, 1993)
pTonB3	pRY107 derivative containing the *tonB3* and its promoter.	This study
pCfrA	pRY107 derivative containing *cfrA* plus its promoter region	(Zeng, *et al.*, 2009)
pTonB3-CfrA	pCfrA derivative containing both intact *tonB3* and *cfrA* genes	This study
pTonB3-CfrA-T	T-easy vector derivative containing the *tonB3-cfrA* locus	This study
pTonB3(tetO)	pTonB3-CfrA-T derivative with *tetO* cassette inserted in *tonB3*	This study
Strains		
C.jejuni		
81-176	Human isolate	(Black, *et al.*, 1988)
JL727	81-176 derivative for which 81-176 was chromosomally complemented with *cee* gene	This study
JL680	81-176 complemented with plasmid pTonB3-CfrA	This study
JL534	81-176 complemented with plasmid pCfrA	This study
11168	NCTC 11168, human isolate	(Parkhill, *et al.*, 2000)
JL709	11168 derivative with *cat* cassette inserted between *cee* and *cj1377c*	This study
JL832	11168 derivative with *tonB3* inactivated by insertion of *tetO* cassette	This study
JL324	11168 derivative, *cfrA::cat*	(Zeng, *et al.*, 2009)
JL612	JL324 derivative with chromosomal complementation of *cfrB* gene. Thus, this strain has genetically repaired CfrB-dependent pathway for FeEnt acquisition	This study
JL845	JL612 derivative, *tonB2$^-$ cfrA$^-$ cfrB$^+$*	This study
JL868	JL845 derivative, *tonB2$^-$ tonB3$^-$ cfrA$^-$ cfrB$^+$*	This study
JL869	JL845 derivative, *tonB3$^-$ cfrA$^-$ cfrB$^+$*	This study
E. coli		
DH5α	F- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (rk−, mk+) *phoA* supE44 thi-1 *gyrA96 relA1 λ*-	Invitrogen
JL48	DH5α/pRK2013, help strain for conjugation.	(Zeng, *et al.*, 2009, Xu, *et al.*, 2010)

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Table 2

Major primers used in this study

Primer	DNA Sequence (5′-3′)[a]	Product size (bp)	Target gene/ operon
Cj1376_F	TTTATCGCTATGGGCTTTGC	1,917	*C. jejuni* cee locus
Cj1376_R	TTGCAAAATGTTTTAAAAGAGCA		
Cm_PacI_F	CCCTTAATTAATGCTCGGCGGTGTTCCTTT	801	Chloramphenicol resistance cassette
Cm_PacI_R	CCCTTAATTAAGCGCCCTTTAGTTCCTAAAG		
TonB3F	TGGCAACACTTTACATAG	1,343	*tonB3* with promoter
TonB3R	CATTGATAGTAGCAGGAG		
rrsF	CTGGAACTCAACTGACGCTAAG	1,900	Ribosomal DNA
rrlR	CTCTTGCACATTGCAGTCCTAC		
CfrB_XbaI_F	GCTCTAGAATGGAGCCTATCAAGAGGCTTAG	2,409	*cfrB* with promoter
CfrB_MfeI_R	GCGCAATTGCCAAGTGCAAAGCCTACCAT		
Erm_MfeI_F	GCGCAATTGAGCTTTGGCTAACACACACG	1,140	Erythromycin resistance cassette
Erm_MfeI_R	GCGCAATTGAATAGGTACACGAAAAACAAGT TAAGG		

[a] Restriction sites are underlined in the primer sequence.

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Table 3

FeEnt utilization system in representative *C. jejuni* and *C. coli* strains.

Strain	*C. jejuni*		*C. coli*
	NCTC 11168	**81-176**	**RM2228**
FeEnt utilization	Yes	No	Not determined
CfrA	+	−	+
CfrB	pseudogene	+	+
TonBl ExbB1/ExbD1	+ +	− −	+ +
TonB2 ExbB2/ExbD2	+ +	+ +	− +
TonB3 ExbB3/ExbD3	+ +	− +	+ +
CeuBCDE	+	+	+
Cee	+	−	+
Source	human	human	Poultry

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Table 4

EZ::TN™ transposon insertion sites in mutants defective of FeEnt acquisition

Mutants	Locus (orientation[a])	Tn location (ORF size in bp)[b]	Annotation/function
1-3D2	*tonB2* (−)	65 (684)	Putative TonB transport protein
4-A1	*tonB2* (+)	555 (684)	Putative TonB transport protein
4-A5	*exbD2* (+)	113 (411)	Putative ExbD/TolR family transport protein
4-A11	*exbB2* (+)	391 (438)	Putative ExbB/TolQ family transport protein
4-D12	*tonB2* (+)	71 (684)	Putative TonB transport protein
5-C3	*cee* (+)	88 (810)	Periplasmic enterobactin esterase
5-D3	*cee* (+)	459 (810)	Periplasmic enterobactin esterase
5-D4	*cee* (+)	330 (810)	Periplasmic enterobactin esterase

ORF, open reading frame.

[a] the orientation of transposon relative to that of disrupted locus. +, same orientation; −, opposite orientation.

[b] The number indicates the nucleotide before which the transposon (Tn) is inserted.