FERROUS AND FERRIC IRON ACQUISITION IN *CAMPYLOBACTER JEJUNI*

By

HEMANT KASHINATH NAIKARE

Master of Veterinary Science in Microbiology
KKV Agricultural University
Maharashtra, India 2000

Bachelor of Veterinary Science
KKV Agricultural University
Maharashtra, India 1998

Submitted to the Faculty of the Graduate College of the Oklahoma State University in partial fulfillment of the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2005

FERROUS AND FERRIC IRON ACQUISITION IN *CAMPYLOBACTER JEJUNI*

Dissertation Approved:

Dr Alain Stintzi
(Dissertation Advisor)

Dr Anthony Confer

Dr Rebecca Morton

Dr Robert Burnap

Dr A. Gordon Emslie

(Dean of the Graduate College)

ACKNOWLEDGEMENTS

I express my heart felt gratitude and appreciation to my mentor Dr. Alain Stintzi for giving me an opportunity to work under his esteemed guidance. I have always admired his passion and perseverance towards his research, and have tried to emulate him in this regard. I would also like to acknowledge my committee members Dr Anthony Confer, Dr Rebecca Morton and Dr Robert Burnap for their constructive criticism throughout the course of my PhD. I wish to express my sincere gratitude to my labmembers Lisa Whitworth, Kiran Palyada, Frederic Poly and Reenu Pandey, who have either directly or indirectly contributed towards my work. I would especially like to thank Lisa for assisting me with the cell culture work, and Kiran for his help in my in vivo characterization studies. I would like to thank Frederic for his contribution towards the bioinformatics sequence analysis. I would like to thank the college of Veterinary medicine for providing me with excellent facilities to pursue my research. I am thankful to Vinay Cheruvu for assisting me with the statistical analysis.

I would also like to thank my wife, Nikita Mirajkar, for her motivation, patience, perseverance and support, without whom I would not be able to complete my graduation on time. She has been highly significant ($p<0.0001$) in the typing of my entire dissertation. I would like to thank my parents for their constant inspiration and support. I would like to extend my thanks to my extended family for their continued support and well wishes.

TABLE OF CONTENTS

Chapter **Page**

LIST OF TABLES

Chapter III

Chapter IV

LIST OF FIGURES

Chapter II

Chapter III

Chapter IV

Figure Page

Chapter I

Introduction

Campylobacter jejuni is a microaerophilic, spiral shaped, motile, gram-negative, enteric food borne pathogen (14). It is a fastidious, facultative intracellular bacterium and requires an oxygen concentration of 3-15% and CO_2 of 3% (14). Biochemically, *C. jejuni* is catalase and oxidase positive and urease negative (26). It does not ferment carbohydrates (14). The optimal growth temperature is 42°C which is also the normal body temperature of poultry, its natural commensal host (17).

C. jejuni is the most common cause of bacterial food-borne enteritis in the industrialized countries (3). Campylobacteriosis is a zoonosis (3). Poultry and poultry products are reported to be the major sources of human infection. *C. jejuni* behaves as a commensal in birds and occurs in exceptionally high numbers (10^8-10^{10} cfu per gram) in the cecum of chickens (12). However, in humans, an infectious dose as low as 500 to 800 organisms have been reported to cause the disease (7). The symptoms associated with campylobacteriosis vary from mild diarrhea to severe colic (25). *Campylobacter* is an important cause of traveler's diarrhea (3). Generally, *Campylobacter* enteritis is a self limiting disease which rarely requires antimicrobial therapy, although one in 1000 infections can lead to a paralytic condition, Guillain- Barré syndrome {GBS} (1, 9) . The annual incidence rate of *Campylobacter* is 13.3 per 100,000 of the US population (29),

and it accounts for 33.4% of laboratory confirmed cases of bacterial gastro-enteritis in the US (29). The high incidence rate of campylobacteriosis necessitates the development of new strategies to combat the disease, and which requires a better understanding of the host-pathogen interactions. The exact mechanism of gut colonization and disease pathogenesis is not well known. However, the availability of the complete genome sequence of *C. jejuni* NCTC11168 in 2000, has led to new opportunities in the investigation of *Campylobacter* pathogenesis. Some of the *Campylobacter* colonization and virulence factors that have been characterized so far include flagella, host cell adherence and invasion, and toxin production (5).

Iron, in addition to having a nutritional role, is also a key environmental signal for controlling the bacterial pathogenesis of most human pathogens (4, 15). Recently, the complex response of *C. jejuni* to iron availability was demonstrated (11, 18). Although iron is abundant in nature, the availability of free iron is very limited in mammalian hosts. Oxidation creates insoluble iron complexes that are not readily available to the bacteria within the host. Bioavailability of iron depends on conditions such as pH and oxygen tension which affect the oxidation state of the iron molecule. Under aerobic conditions at neutral to alkaline pH, iron is in the insoluble form as Fe (III), and its bioavailability is limited to 10^{-18} M (22). In the mammalian host its bioavailability is 10^{-24} M as majority of Fe(III) is tightly bound to iron-binding and transport proteins such as hemoglobin, transferrin, lactoferrin and ferritin (22). These levels are far below the minimum requirements of 10^{-7} M for the bacterial growth. Therefore, microorganisms have evolved complex mechanisms to acquire iron and maintain its homeostasis.

The enteric pathogenic bacteria utilize several methods for high affinity iron acquisition. The two most commonly used methods are the direct utilization of host iron sources such as heme, hemoglobin, transferrin and lactoferrin and synthesis and transport of siderophores (29). Siderophores are low molecular weight compounds that chelate Fe(III) with sufficient affinity to remove it from host compounds (24) . Only a few strains of *C. jejuni* have been shown to produce siderophores and these siderophores are uncharacterized (6). The *C. jejuni* genome lacks the genetic machinery required for the biosynthesis of enterobactin which is an *E. coli* siderophore (19). However, all the strains of *Campylobacter* tested were able to acquire iron from enterobactin. Ferric- siderophore complexes exceed the diffusion limit of the outer membrane of gram-negative bacteria; thus energy dependent transport systems are required for their efficient acquisition. There is no known source of energy available at the outer membrane, and the proton motive force (PMF) of the cytoplasmic membrane is harnessed as the energy source (20) via the TonB system, consisting of three proteins: TonB, ExbB, and ExbD. The TonB system transduces the cytoplasmic membrane proton motive force (PMF) to the active transport of nutrients through outer membrane receptors. *E. coli* and most of the other gram-negative bacteria contain a single TonB system (2, 20) whereas *V. cholerae* has two distinct TonBs which contribute to its ability to use a variety of iron sources to perform specific as well as redundant functions (16). Based on the genome sequence of *C. jejuni* NCTC 11168, there are three putative TonB systems (19). These are not closely mapped on the chromosome, and their exact role is not clear. We hypothesize that the presence of

three TonBs in *C. jejuni* may contribute to its ability to utilize varied iron sources under different environmental conditions.

The complete genome sequence of *C. jejuni* NCTC11168 suggests the presence of several iron acquisition systems, some of which have been partially/completely identified: a complete hemin uptake system [ChuABCD] (27), an enterochelin transport system [CeuBCDE] (23, 27), a ferric enterobactin uptake receptor [CfrA] (10, 18), ferrichrome uptake system [CfhuABD] (8), and three putative TonB/ ExbB/ ExbD complexes (27). Additionally, a putative ferrous iron transporter protein [FeoB] has been identified (21). The FeoB-mediated ferrous iron transport has been demonstrated to be critical for growth of *E. coli* (13) and *H. pylori in vivo* as well as *in vitro* (28). However, the role of these iron acquisition systems as virulence/colonization determinants in *Campylobacter* pathophysiology is not completely understood. Also, the intracellular growth environment with respect to the availability of specific nutrients and the nature of the iron sources (whether ferrous or ferric) available to the different strains of *C. jejuni* in the cytoplasm of the infected host cells within the gut is not known. Based on the above background information, I propose to accomplish the following objectives:

1. Characterize FeoB mediated Fe (II) iron uptake and
2. Characterize TonB dependent Fe(III) iron uptake in different strains of *C. jejuni* and examine their role in *Campylobacter* pathophysiology.

References

1. Ang, C. W., P. G. Noordzij, M. A. de Klerk, H. P. Endtz, P. A. van Doorn, and J. D. Laman. 2002. Ganglioside mimicry of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity in rabbits. Infect Immun 70:5081-5.

2. Braun, V. 1989. The structurally related exbB and tolQ genes are interchangeable in conferring tonB-dependent colicin, bacteriophage, and albomycin sensitivity. J Bacteriol 171:6387-90.

3. Butzler, J. P. 2004. *Campylobacter*, from obscurity to celebrity. Clin Microbiol Infect 10:868-76.

4. Byers, B. R., and J. E. Arceneaux. 1998. Microbial iron transport: iron acquisition by pathogenic microorganisms. Met Ions Biol Syst 35:37-66.

5. Crushell, E., S. Harty, F. Sharif, and B. Bourke. 2004. Enteric *Campylobacter:* purging its secrets? Pediatr Res 55:3-12.

6. Field, L. H., V. L. Headley, S. M. Payne, and L. J. Berry. 1986. Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. Infect Immun 54:126-32.

7. Frost, J. A. 2001. Current epidemiological issues in human Campylobacteriosis. Symp Ser Soc Appl Microbiol:85S-95S.

8. Galindo, M. A., W. A. Day, B. H. Raphael, and L. A. Joens. 2001. Cloning and characterization of a *Campylobacter jejuni* iron-uptake operon. Curr Microbiol 42:139-43.

9. Gilbert, M., P. C. Godschalk, M. F. Karwaski, C. W. Ang, A. van Belkum, J. Li, W. W. Wakarchuk, and H. P. Endtz. 2004. Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barre syndrome, by horizontal exchange. Infect Immun 72:1162-5.

10. Guerry, P., J. Perez-Casal, R. Yao, A. McVeigh, and T. J. Trust. 1997. A genetic locus involved in iron utilization unique to some *Campylobacter* strains. J Bacteriol 179:3997-4002.

11. Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells. 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology 151:243-57.

12. Jacobs-Reitsma, W. F., H. M. Maas, and W. H. Jansen. 1995. Penner serotyping of *Campylobacter* isolates from poultry, with absorbed pooled antisera. J Appl Bacteriol 79:286-91.

13. Kammler, M., C. Schon, and K. Hantke. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J Bacteriol 175:6212-9.

14. Kelly, D. J. 2001. The physiology and metabolism of *Campylobacter jejuni* and *Helicobacter pylori*. Symp Ser Soc Appl Microbiol:16S-24S.

15. Litwin, C. M., and S. B. Calderwood. 1993. Role of iron in regulation of virulence genes. Clin Microbiol Rev 6:137-49.

16. Mey, A. R., and S. M. Payne. 2003. Analysis of residues determining specificity of *Vibrio cholerae* TonB1 for its receptors. J Bacteriol 185:1195-207.

17. Newell, D. G. 2001. Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models. Symp Ser Soc Appl Microbiol:57S-67S.

18. Palyada, K., D. Threadgill, and A. Stintzi. 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol 186:4714-29.

19. Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M.

Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-8.

20. Postle, K., and R. J. Kadner. 2003. Touch and go: tying TonB to transport. Mol Microbiol 49:869-82.

21. Raphael, B. H., and L. A. Joens. 2003. FeoB is not required for ferrous iron uptake in *Campylobacter jejuni*. Can J Microbiol 49:727-31.

22. Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol 54:881-941.

23. Richardson, P. T., and S. F. Park. 1995. Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system. Microbiology 141 (Pt 12):3181-91.

24. Schmitt, M. P., and S. M. Payne. 1988. Genetics and regulation of enterobactin genes in *Shigella flexneri*. J Bacteriol 170:5579-87.

25. Stintzi, A. 2003. Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. J Bacteriol 185:2009-16.

26. van Vliet, A. H., and J. M. Ketley. 2001. Pathogenesis of enteric *Campylobacter* infection. Symp Ser Soc Appl Microbiol:45S-56S.

27. van Vliet, A. H., J. M. Ketley, S. F. Park, and C. W. Penn. 2002. The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. FEMS Microbiol Rev 26:173-86.

28. Velayudhan, J., M. A. Jones, P. A. Barrow, and D. J. Kelly. 2004. L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. Infect Immun 72:260-8.

29. Wooldridge, K. G., and P. H. Williams. 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiol Rev 12:325-48.

Chapter II

Review of Literature

1 Iron

Iron plays a pivotal role in the metabolism of almost all forms of life and occupies a unique position in biological systems. It is an essential element for almost all living organisms, with the possible exception of only *Lactobacillus* species and *Borrelia burgdorferi* that utilize manganese and cobalt as biocatalysts in place of iron (35, 74, 102). Iron is an important co-factor of many enzymes and is involved in a wide variety of biological mechanisms such as oxygen transport, electron transfer, photosynthesis, amino acid biosynthesis and DNA synthesis (73).

1.1 Availability of Iron

Although abundant on earth, the bioavailability of iron is very limited in most environments. In aerobic environments and at neutral to alkaline pH, iron occurs in the oxidized ferric form, Fe(III). The ferric form aggregates into insoluble, oxy-hydroxide polymers. This insoluble ferric iron is not readily available for bacteria unlike the Fe(II) form, which is soluble (98). Paradoxically, although iron is essential for bacteria, excess iron is toxic. Iron catalyzes the formation of harmful hydroxyl anions and radicals via the

Haber-Weiss and Fenton reactions. The ferrous iron can activate the Fenton reaction and react with hydrogen peroxide which is formed under aerobic conditions.

1.2 Haber-Weiss Reactions

The traces of ferric iron can react with H_2O_2 to produce Fe (II) and generate superoxide anion.

$$Fe\ (III) + H_2O_2 \longrightarrow Fe\ (II) + O_2^- + 2H^+$$

In another reaction, the Fe (III) iron can react with superoxide anion to form ferrous iron and molecular O_2

$$Fe\ (III) + O_2^- \longrightarrow Fe\ (II) + O_2$$

The toxicity of the O_2^- and H_2O_2 involves their conversion to $OH^{\cdot}$ radical (102). Therefore, in human and animal tissues, to avoid the generation of free radicals, iron is complexed into hemoglobin and stored intracellularly in ferritin, or is chelated by transferrin in serum and by lactoferrin at mucosal surfaces (35). Thus, iron homeostasis is strictly regulated. Consequently, there is restriction of free iron availability to support bacterial growth.

1.3 Fenton Reaction

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + OH^{\bullet}$$

The Fenton reaction produces highly reactive hydroxyl anions and hydroxyl radicals that are very harmful for most of the macromolecules (102).

2 Iron Transport in gram-negative Bacteria

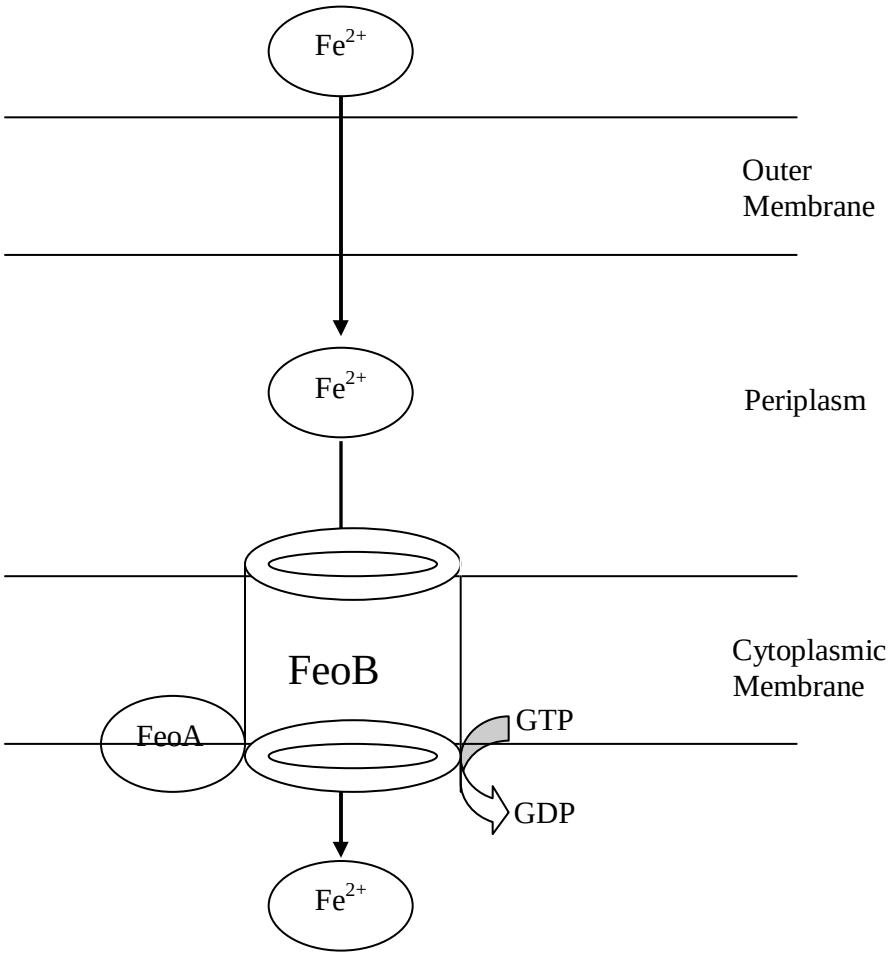
Gram-negative bacteria have developed several mechanisms to acquire iron from various sources available in their respective habitats and have to compete fiercely with the normal microbiota within their habitat. The cell envelope of Gram-negative bacteria comprises the inner cytoplasmic membrane (CM), the peptidoglycan rich periplasm, and the outer membrane (OM). The outer membrane confers protection to the cell and selectively permeates uptake of nutrients. The proteins within the OM either provide porin channels for passive solute diffusion or mediate active transport (63). For example, ferrous iron is mostly soluble under anaerobic conditions and can passively diffuse through the porins in the OM, whereas, ferric complexes such as transferrin, heme or lactoferrin are too large to be transported by porins and need to be actively transported (63). Broadly based upon the oxidation state of iron, iron sources can be categorized as ferrous and ferric.

3 Fe(II)

Fe (II) iron is the major form of iron under anaerobic and reducing conditions or at acidic pH. Fe (II) iron uptake is also important in the presence of oxygen since many bacteria can reduce extracellular ferric iron (98). The ferrous iron is highly soluble, and a small molecule of less than 600 Da, may diffuse through outer membrane porins. There is no evidence for the involvement of any specific outer membrane receptor in this process.

Once inside the periplasm, the ferrous iron is transported through the cytoplasmic membrane via the "FeoB" protein (2). FeoB homologs are found in genomes of Archae, gram-positive and gram-negative bacteria (98). The FeoB protein is highly conserved in many species of gram-negative bacteria. The crystal structure of FeoB has not yet been determined in any bacteria. The FeoB protein contains a G-Protein domain (39, 60), suggesting that FeoB energizes the transport of ferrous iron through the cytoplasmic membrane via GTP hydrolysis. Refer to Figure 1 for the schematic diagram of ferrous iron transport in gram-negative bacteria.

Figure 1: Schematic Diagram of Ferrous Transport in gram-negative Bacteria

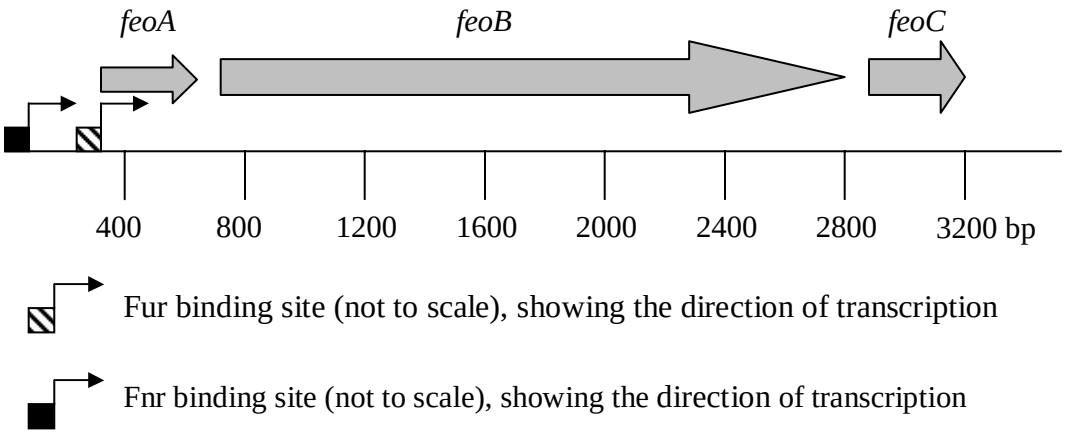


{Source: Modified from Velayudhan *et.al* (2000) Molecular Microbiology 37:274-286}

3.1 Ferrous Iron Uptake in *E. coli*

The first bacterial ferrous iron transport system to be identified was the high affinity Feo transport system of *E. coli* (49), encoded by the *feoABC* operon. The *feoA* encodes a small protein of 75 amino acids, while *feoB* encodes a membrane protein of 773 amino acids. The *feoC* was initially overlooked, but was recently reported to be present in the operonic frame. It encodes a protein of 78 amino acids in length, and is found only in members of the Enterobacteriaceae (49). Figure 2 is the schematic diagram of the *feoABC* operonic structure in *E. coli*. Initially it was thought that the transport of ferrous iron was driven by ATP hydrolysis. More recently FeoB has been shown to be similar to eukaryotic and prokaryotic G-proteins (60). There are 4 of the 5 GTPase characteristic signature motifs in the FeoB of *E. coli*. Based on mutational studies, it has been proposed that GTPase activity is necessary for iron uptake. However, the mechanism of transport and the precise function of FeoB still need to be determined. Similarly, the exact role of FeoA and FeoC in the uptake is not known, and they might play the role of modulators by changing the properties of the GTPase domain of FeoB. Hence, they may speed the uptake process and satisfy the iron needs of the cell (60).

Figure 2: *E. coli feoABC* Operonic structure (source Ferrous iron transport)



{Source: Iron transport in bacteria (2004) Chapter 12. Ferrous iron transport Page 179}

3.1.1 Regulation of FeoB in *E. coli*

It is evident in *E. coli* that ferrous iron uuptake is repressible by iron. There are 2 protein regulators of ferrous uptake, namely, Fur and Fnr.

The *fur* gene is the iron uptake regulator gene and its binding site, known as the Fur box, is present upstream of the *feoB* gene. Binding of the *fur* gene to the Fur box has been demonstrated *in vivo*. In addition, the ferrous iron uptake system is over expressed in a *fur* mutant (3).

The Fnr activates genes required for optimal growth under anaerobic conditions. It has been demonstrated that regulation of *feo* by Fnr likely favors ferrous iron uptake under anaerobic conditions (3, 49).

3.1.2 FeoB and Pathogenicity in *E. coli*

Enteric pathogens, upon their entry into the host, have to colonize, survive and replicate in the GI tract, and must efficiently acquire iron under extremely low iron conditions. It has been shown that *feoB* mutants in *E. coli* were less efficient in their ability to colonize the intestine as compared to the wild type strain (91). *E. coli* has been shown to possess *ribA* and *ribB* genes which are involved in riboflavin biosynthesis, and the riboflavin thus synthesized, is involved in ferric reduction activity (46). CorA, a magnesium transporter, has been shown to transport ferrous iron in the absence of magnesium (38). It has been demonstrated that *E. coli feoB* mutants take up less ferrous iron than the wild type in the presence of 1 mM magnesium (38). Thus, CorA appears to be a non-specific ferrous transporter in *E. coli.*

3.2 Ferrous iron transport of *Helicobacter pylori*

H. pylori is a microaerophilic, gram-negative bacterium, and a major cause of gastritis and adenocarcinoma of the stomach (7, 8, 50-53). An Fe (II) transporter 'FeoB' exists in *H. pylori* (1). It is 29% identical to the *E. coli* FeoB protein. There is no evidence of FeoA homolog in *H. pylori* (1). Iron transport in *feoB* mutant was 10 fold lower than that in the wild type strain, and complementation of the mutant fully restored its high affinity ferrous transport (101). A *feoB* mutant was more sensitive to other metal toxicities such as zinc, nickel and manganese than the wild type strain (101). The increased sensitivity of the *feoB* mutant to other metals is likely due to increased expression of other transport systems, leading to an increase in the accumulation of potentially toxic metals. Also, the *feoB* mutant was unable to colonize the gastric mucosa of mice, indicating its importance in establishing colonization in the low pH, low O_2 environment of the stomach (101). Similar to *E. coli*, it has been demonstrated that the magnesium transporter *CorA* catalyzes low affinity ferrous iron uptake in *H. pylori* (101). The *H. pylori g*enome also possesses an iron regulated *ribBA* gene which encodes the riboflavin biosynthesis protein (24, 105). The riboflavin synthesized under iron restricted conditions was shown to be excreted in the culture medium where it acts as a reductant. Disruption of *ribBA* in *H. pylori* eliminated ferric reduction activity (24, 105).

3.3 FeoB dependent Fe (II) uptake in *Salmonella* species

In *S. typhimurium*, Fe (II) iron is transported by a high affinity transport system encoded by the FeoABC system (96). In addition to the transporter, *S. typhimurium* possesses a magnesium transport system encoded by CorA protein which might also contribute to the

non-specific transport of iron into the cell, as well as the non-specific transport of other essential rare metals like cobalt, manganese and nickel, which are required in trace amounts by the cell (38). Interestingly, *S. typhimurium feoB* mutant was shown to be out competed by the wild type strain during mixed colonization in the mouse model (96). However, the disruption of FeoB did not attenuate the *feoB* mutant for oral or intra peritoneal infection in mice (96). In fact, the *feoB* gene was shown to contribute to the bacterial growth at intra-intestinal sites, while the *tonB* gene (another iron transport system) was found to be required for the subsequent colonization of Peyers patches of mesenteric lymph nodes. Consequently, different iron transport systems might be required at different stages of mice infection (9, 47, 80, 96).

3.4 Ferrous iron transport in *Shigella* species

3.4.1 *Shigella flexneri*

S. flexneri is a facultative intracellular bacterium that causes dysentery in humans (78, 103). It possesses several iron acquisition systems comprised of siderophore mediated ferric transporters, high affinity ferrous transporter "FeoB", and the SitA transport system, which transports ferrous iron and manganese (83, 104) . The *sit* genes are in an operonic frame *sitABCD*, and are located in a pathogenicity island. Expression of SitA is induced in the eukaryotic intracellular environment, which is suggestive of its role in the survival or growth of *Shigella* within the eukaryotic cells (83, 104). The FeoB system also contributes towards *Shigella's* intracellular survival or growth in the eukaryotic cells (83, 104).

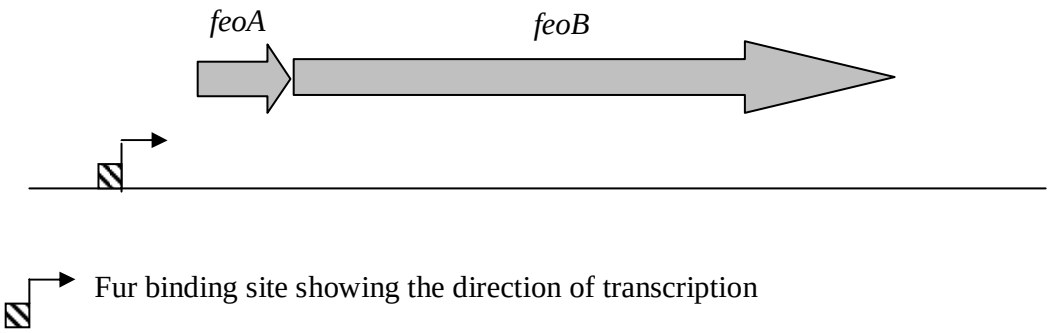
3.4.2 *Shigella dysenteriae*

S. dysenteriae is a facultative intracellular human pathogen and causes bacillary dysentery (78). Its virulence is dependent on its ability to invade colonic epithelial cell, escape from phagosomes, multiply intracellularly, and spread to adjacent cells (78). It is likely that FeoB transporter contributes towards its virulence machinery since a TonB independent iron transport system was shown to exist in *S. dysenteriae* (78).

3.5 Ferrous iron transport in *Campylobacter jejuni*

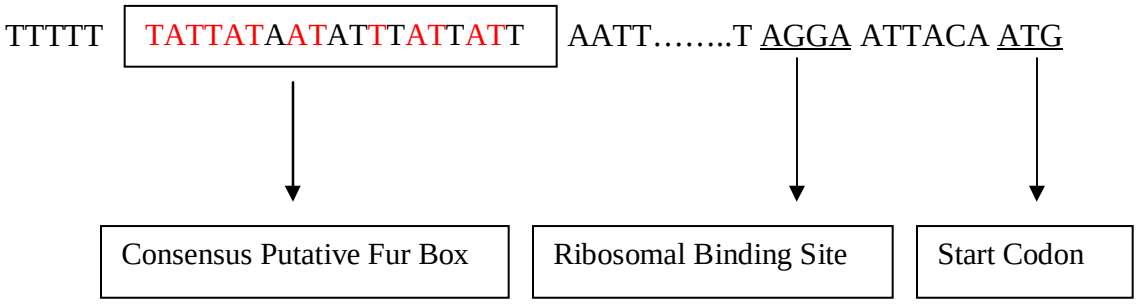
The importance of iron acquisition and metabolism for the successful colonization of the host has been demonstrated for many pathogens (77). *C. jejuni's* lifestyle in the rabbit gut was recently studied and iron acquisition genes were identified to be important for gut colonization (88). The genome sequence of *C. jejun*i NCTC11168 reveals the presence of a FeoB homolog (Cj1398), which is approximately 1.8 Kb in size and encodes a 70 KDa cytoplasmic membrane protein (70). *C. jejuni* FeoB shares 50 % and 29 % amino acid identity with *H. pylori* and *E. coli* FeoB respectively. Cj1398 appears to be in an operonic structure with Cj1397 (Figure 3) (70, 98). However, this has not yet been experimentally demonstrated. Cj1397 encodes a putative protein of 74 amino acids and bears 16% identity to the *E. coli* FeoA (98). However, its role in iron transport is not yet known.

Figure 3: Cj 1397 (*feoA* homolog) - Cj 1398 (*feoB* homolog) Operon



The promoter region of Cj1398 contains a putative "Fur" box (98). Therefore, the expression of FeoB is likely Fur regulated. The putative Fur binding site upstream of *C. jejuni* FeoB is shown in Figure 4.

Figure 4: *C. jejuni* FeoB Putative Fur Box



{Source: AHM van Vliet *et al.* FEMS Microbiology Reviews 26 (2002) Page 182}

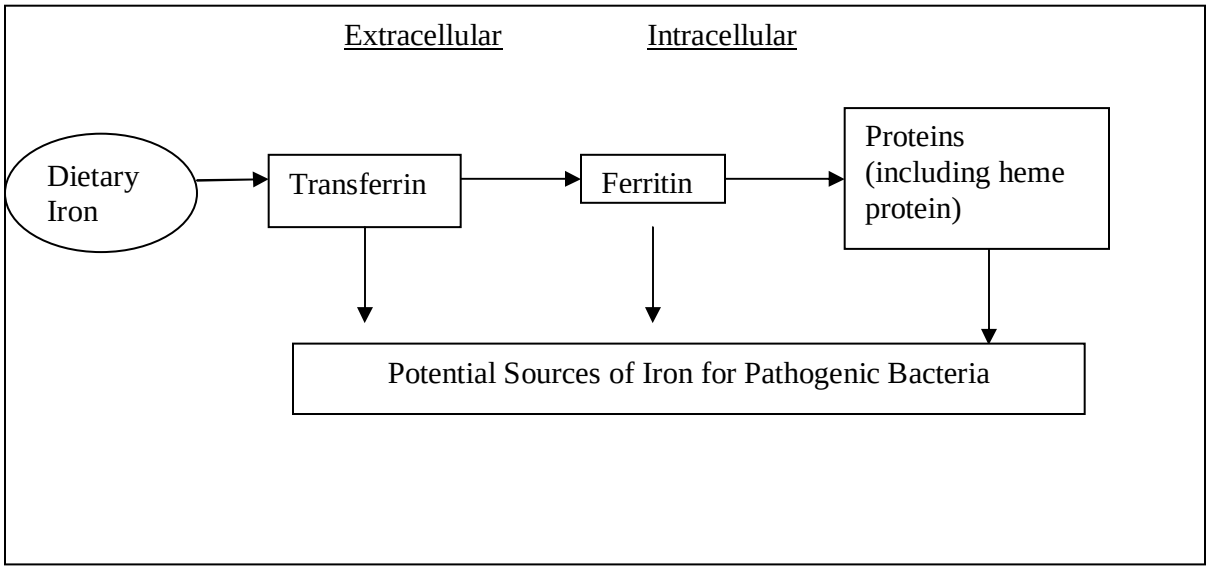
The *fur* mutant in *C. jejuni* was shown to be significantly affected in colonization of the chicken gut (68). Raphael *et al* characterized the *C. jejuni feoB* genes from the strains M129, F38011, and RM1221 (76). They reported that FeoB did not play a role in ferrous iron transport in *C. jejuni*. They did not observe any significant difference in invasion and intracellular survivability of the *feoB* mutant and the wild type. Also, they found the *corA* gene to be not involved in ferrous iron transport, whereas *corA* has been demonstrated to be involved in low affinity ferrous iron transport in *H. pylori, E. coli* and *Salmonella* (39, 101). Raphael *et al*. reported FeoB to be not iron regulated since the *feoB* transcripts were observed under iron rich and iron starved conditions. Their findings are in contrast to the recent *in vitro* reports which demonstrated the iron acquisition systems to be repressed under iron rich condition and de-repressed under iron starved condition (44, 68). Interestingly, the *C. jejuni feoB* gene was found to be up-regulated *in vivo* (89). The contradictory evidence pertaining to the involvement of FeoB in ferrous iron uptake can be attributed to the different strains used in characterization and the growth conditions prior to performing the assays. Till date, there has been no *in vivo* characterization of the *feoB* mutant of *C. jejuni* in any animal models of colonization.

4 Fe (III)

In an aerobic neutral pH environment, the concentration of free ferric iron is only about 10^{-18} M since most of the iron occurs in the insoluble $Fe(OH)_3$ form (77). Therefore, the concentration of free ferric iron available for biological processes is far below the minimum requirement of the cell (102). Consequently, pathogens have evolved specific

mechanisms for the acquisition of iron form their hosts. The principal host sources of iron which may be accessed by specific bacterial pathogens are outlined in Figure 5.

Figure 5: Potential Iron sources for Bacterial Pathogens



{Source: Ratledge and Dover (2000) Annual Reviews of Microbiology 54:881-941}

Iron acquisition in gram-negative bacteria has revolved around 2 strategies. These are as follows:

1) Direct contact of the bacterium with the source of iron (usually transferrin or individual iron proteins such as heme). Once the contact is established, there is direct removal of iron, and uptake occurs (35, 77, 102).
2) An alternative strategy adopted by many gram-negative bacteria is to synthesize a high-affinity ferric iron chelator or to utilize the chelators produced by other bacteria to efficiently steal and capture the iron from the host iron-binding proteins. Such compounds are known as siderophores (35, 77, 102).

4.1 Iron Sources from Host Proteins

4.1.1 Transferrin (Tf) and Lactoferrin (Lf)

These are monomeric glycoproteins of ~ 80 KDa and bind two ferric ions per molecule. The affinity constant for Fe (III) ion is ~ 10^{23} M^{-1} and that for Fe (II) ion is only ~ 10^{3} M^{-1} (19, 20). Tf serves two purposes: Iron transport and protection against iron toxicity. Lf confers protective function as an iron chelator. X-ray crystal structures of both of these sources have been reported (48, 56). Many bacterial pathogens have been documented to use these iron sources (15, 92, 106).

4.1.2 Ferritins

These are iron storage proteins consisting of 24 sub-units assembled into a hollow sphere with a diameter of 120 Å and are filled with several hundred thousand of Fe(III) ions. However, there is a lack of evidence of the use of ferritins as an iron source by bacteria (17).

4.1.3 Heme

Heme is an iron protoporphyrin IX molecule. It is a prosthetic group of many enzymes. Although it is scarcely found free, it is used as an iron source by many bacterial species. Heme is required in nM range while iron requirement ranges in µM concentrations for bacteria (102).

4.1.4 Hemoglobin (Hb)

It is a tetramer of two α and two β chains and each subunit binds a heme molecule. Hb is present in red blood cells and functions as an oxygen transporter. Many pathogens as well as non pathogenic bacterial species have heme-hemoglobin uptake systems such as *Vibrio cholerae*, *Shigella dysenteriae*, *Yersinia pestis*, *Haemophilus influenzae*, and others (77, 102).

4.1.5 Hemopexin

This is a 60 KDa glycoprotein. Although several bacteria have been shown to bind and/or to use heme-hemopexin, it has not been demonstrated whether a direct utilization exists (77, 102).

4.2 Siderophores

These are low molecular weight iron chelating compounds that are secreted into the environment (64, 66). The first siderophore to be identified and characterized was ferrichrome, a reddish brown iron binding compound produced by the fungus *Ustilago*

(55). The chelating power of the siderophores for iron is very strong, such that it can remove iron attached to molecules like Tf and Lf, but not from heme proteins. The dissociation constant (Ks) ranges from 10^{22} M to 10^{50} M (102). The number of different siderophores produced by bacteria, yeast and fungi is more than 500 (102). However, not all siderophore producing microbes are pathogenic. Siderophore biosynthesis is invariably derepressed when cells are grown under iron limiting conditions (65, 66).

4.3 Classification of Siderophores

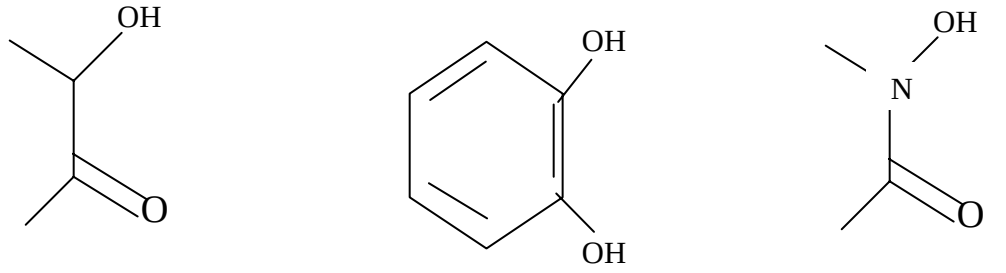
The 3 major classes of siderophores based on chemical structure and metal binding functionality are: catecholates, hydroxamates and hydroxycarboxylates (102).

Figure 6 shows the principal ferric iron chelating groups found in siderophores. There are two common features among all the structurally diverse siderophores:

i) Presence of hard donor atoms (usually oxygen, occasionally nitrogen or sulfur)

ii) Formation of thermodynamically stable, high spin ferric species.

Figure 6: Principal Functional Groups found in Siderophores



α-Hydroxycarboxylic acid Catechol Hydroxamic acid

{Source: Raymond and Dertz (2004) Chapter1 Pages 1- 17, Iron transport in bacteria, ASM Press}.

4.3.1 Catecholate Siderophores

4.3.1.1 Enterobactin

This is the best characterized catecholate. It has a trilactone backbone and possesses a three-fold symmetry. It is composed of three 2, 3-dihydroxybenzoic acid groups, each appended to an L-serine group (77). The *E. coli* enterobactin gene cluster contains 6 genes (*entA* to *entF*) involved in its biosynthesis and 7 genes involved in its utilization. These 7 genes encode for a ferric-enterobactin transporter system (*fepA* to *fepG*) and an esterase gene, which hydrolyses the molecule at its three ester linkages releasing iron in the cytoplasm (36, 37). The Enterobactin has the highest stability constant for iron among all the known siderophores (77). The enterobactin breakdown products and the enterobactin precursor (DHB) can also serve as secondary siderophores to meet the iron requirements of the cell (36, 37, 102).

4.3.1.2 Salmochelins

These are catecholate-siderophores consisting of 2 or 3 dihydroxybenzoic acid moieties linked by glucose residues. The proteins involved in the synthesis and transport of salmochelins are encoded in the *iro* locus. With the exception of *S. bongori*, all *Salmonella* strains synthesize salmochelins and have the *iro* genes. The *iro* locus is also present in *Shigella dysenteriae* type I and some pathogenic *E. coli* strains (5, 41).

4.3.2 Hydroxamate Siderophores

These are produced by gram-positive and gram-negative bacteria and fungi. Typically there are two or three hydroxamate groups per molecule. They are observed with either linear or cyclic conformations. The ferrichrome and aerobactin are the most common hydroxamates (102).

4.3.2.1 Ferrichrome

This is a cyclic hexapeptide with 3 contiguous glycine residues and 3 molecules of N-acyl-N-hydroxyornithine. It is used as an iron source by fungi, gram-positive and negative bacteria. Of gram-negative bacteria, *E. coli, Salmonella* species and *V. cholerae* have well characterized ferrichrome transport (77).

4.3.2.2 Aerobactin

Enteric pathogens like *E. coli, Shigella, Yersinia, Klebsiella* and *Salmonella* are known to produce aerobactin (77, 102)**.** The aerobactin backbone is comprised of two molecules of lysine, two acetyl groups derived from acetyl CoA and one molecule of citrate, which is the central linker. The *iucABCD* operon is responsible for the biosynthesis of aerobactin in *E. coli.* The *iutA* encodes an outer membrane receptor ~ 77 KDa. There is evidence that bacteria which produces or utilizes both aerobactin and enterobactin, such as *E. coli*, utilize these siderophores under different conditions (13). The enterobactin is used to acquire iron from Tf, whereas aerobactin is utilized to obtain iron preferentially from ferritin within macrophages (13).

4.3.3 Hydroxycarboxylates

This class of ferric siderophores is a five membered heterocyclic compound that arises by cyclization of cysteine, serine or threonine side chains during siderophore elongation on NRPS assembly lines. NRPS is non ribosomal peptide synthetase. It has specialized domains that link amino acids via thio ester intermediates. The domains required for peptide bond formation are adenylation, peptidyl carrier protein and condensation domains, which are physically tethered to each other in repeats. Examples of hydroxycarboxylates include anguibactin, pyochelin, yersiniabactin and vibriobactin (102).

4.4 Synthesis and Excretion of Siderophores

Genes that encode for siderophore biosynthesis are iron regulated. They are generally found to be in a cluster with genes involved in siderophore uptake. Siderophores are usually assembled by non ribosomal cytoplasmic peptide synthetase (102)

Once the siderophore is synthesized and utilized, it needs to be eventually excreted. The mechanism of excretion is not clearly understood. Currently, data is only limited to a single report wherein the gene, *EntS,* involved in the excretion of enterobactin, was determined to be located within the enterobactin biosynthesis gene cluster. EntS encodes a membrane protein and is directly involved in enterobactin export via a proton-motive force dependent efflux pump (30).

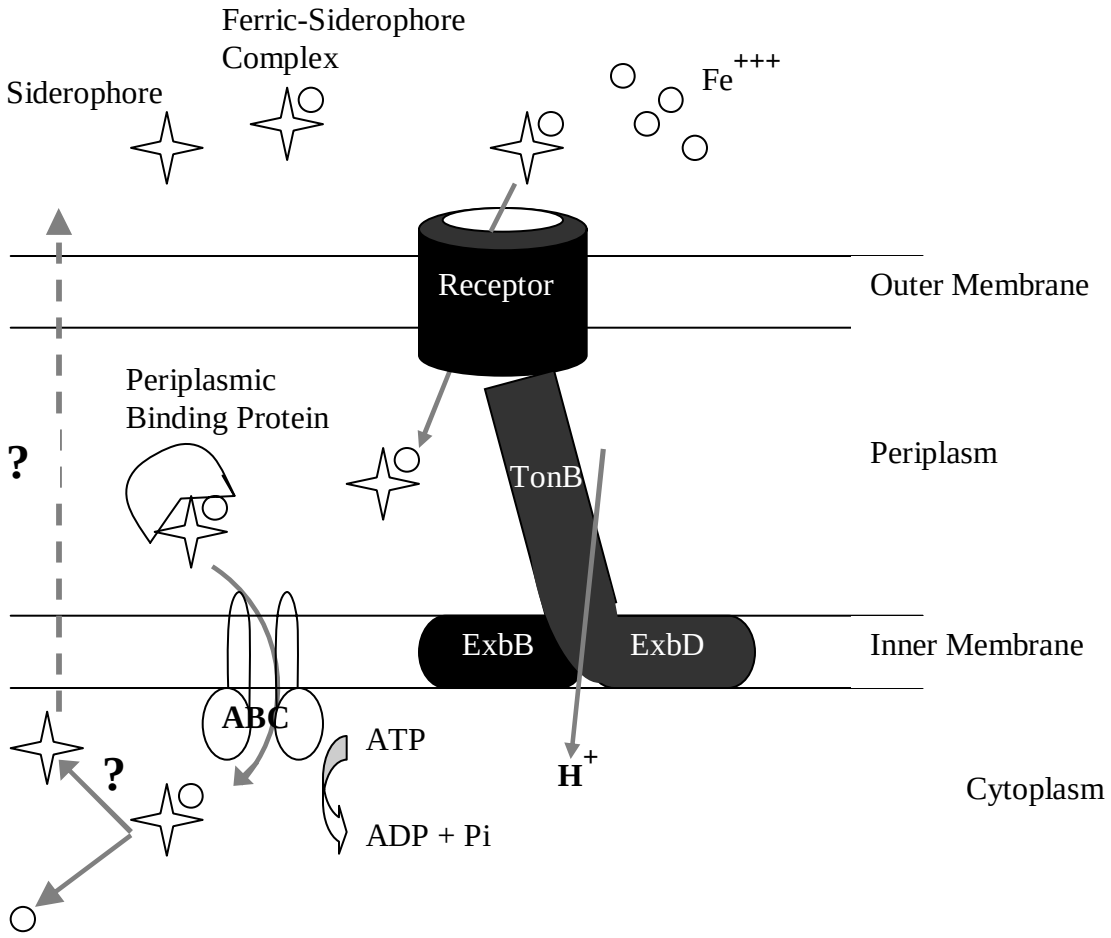
4.5 Ferric-Siderophore uptake

Ferric-siderophores and heme molecules are too big to diffuse through the restricted diameter of porins (35, 63). In addition, these compounds must be efficiently captured at the cell surface by outer membrane receptors. These receptors are highly specialized and specific and effectively concentrate the ligand (heme, ferric-siderophores) at the cell surface. In the case of heme, only the prosthetic group is transported across the OM. The mechanism for stripping of the prosthetic group on the extracellular surface of the OM and the exchange of holo-and apo-proteins is not well known (102). The subsequent translocation of these molecules into the periplasm is an active process which works against the concentration gradient and is energized by the proton motive force provided by the functional TonB complex (57, 58) Please refer to Figure 7 for the schematic representation of TonB dependent ferric iron transport in gram-negative bacteria.

5 Outer Membrane Receptors (OMR)

Until recently, three OMRs have been crystallized, namely, FhuA, FepA and FecA (14, 18, 26, 28, 59, 107). All these receptors belong to *E. coli*. FepA is the receptor for enterobactin. FhuA is the receptor for ferrichrome and FecA is the receptor for ferric dicitrate. These OMRs share some common characteristics. They have a low level of primary sequence homology in their N termini, and their transport function depends on the TonB complex. The OMRs have 5 conserved residues, together known as the TonB box at their N-termini (10, 23).

Figure 7: Schematic representation of TonB dependent ferric iron transport in gram-negative bacteria.



{Source: Modified from Annual Reviews of Microbiology, 2004.58:611-647}

The receptor structures have a “plug and a beta barrel” organization (77). N termini of the OMR that constitute the plug is folded inside the beta barrel and anchored to the OM. The beta barrel consists of 22 beta strands. The structure is stabilized by the formation of hydrogen bonds and salt bridges between the plug and inner surface of the beta barrel. The plug has a “floor” which mainly consists of beta strands. The substrate sits at the top of the plug. Substrate binding triggers conformational changes in the apices of the plug, which triggers movement in the periplasmic side and allows the functioning of the TonB complex(27).

6 TonB

‘Ton’ stands for T-one and dates back to the 1940’s classical experiment wherein it was demonstrated that *tonB* mutations confer resistance to T1 Bacteriophage. Hence, the mnemonic “ton” for T-1 (12). The TonB complex consists of three proteins TonB, ExbB and ExbD (16). This complex interacts at the cytoplasmic membrane (CM) and transduces the energy of the proton motive force generated at the CM to the OMR for allowing substrate internalization into the periplasmic space. The substrate internalized includes iron, its chelates, heme and also vitamin B_{12} (16). Although it is evident that the CM protein TonB is required for this process, the exact mechanism for transduction of energy is still incompletely understood (102). Transport over the CM is subsequently mediated by ABC transporter. TonB activity has been related to OM transport of Fe (III), Fe (III)-siderophore complex, heme and vitamin B_{12} (11). The TonB system is considered as a target for antibiotics since it plays a role in the specific permeability of gram-negative cell envelope (33). It is evident that upon substrate binding by the receptor, the

TonB protein binds to the membrane receptor, TonB interacts preferentially and directly with ligand loaded receptors (75). Also, it has recently been recognized that certain TonB dependent transporters contain an additional domain at the N terminus (107). This interacts with cytoplasmic membrane regulatory protein and a cytoplasmic sigma factor to induce transcription of iron transport genes. Based on biophysical and crystallographic evidence, these TonB dependent iron transporters bind to both, iron free and iron loaded ligands, however, it is only the ferric loaded ligand that is transported into the periplasm (81, 86, 107).

6.1 Conservation of TonBs

The TonB membrane domain is likely to be conserved over a range of gram-negative bacteria. It has been shown that *E. coli tonB* mutant was complemented with *tonB* gene from *Salmonella* or from *Serratia* , but not from *Pseudomonas putida* or *V. chloerae tonB1* gene (6, 31, 67, 87, 96). Interestingly, *V. cholerae tonB2* could complement in an *E. coli tonB* mutation (87). This differential ability to function in *E. coli* suggest that the targets to which the complex delivers energy in *E. coli* differs from the target of the *V. cholerae tonB1* (87).

6.2 Single TonB systems

6.2.1 TonB complex in *E. coli*

E. coli has a single TonB complex, which consists of 239 amino acids and is ~ 26 KDa in size, the bulk of which occupies the periplasmic space, where it associates with both CM and OM components (10). A *tonB* mutant in *E. coli* strain CFT073 failed to use heme as an iron source or to utilize enterobactin and aerobactin, suggesting that heme and siderophore mediated iron transports are TonB dependent (95). Further, the *tonB* mutant of uro-pathogenic *E. coli* strain was found to be significantly reduced in virulence in a mouse model of urinary tract infection (95). In addition, the *tonB* mutant failed to compete with the wild type for colonization and to infect the kidneys in a mouse model (95).

6.2.2 TonB Complex in *Salmonella* species

Most of the iron transport studies have been conducted with *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi. No single siderophore is found to be essential for *Salmonella* virulence. However, TonB dependent transport is required (54, 96). A *S. enterica* serovar Typhimurium *tonB* mutant was found to be attenuated for virulence by intragastric route (96). However, the mutant was as virulent by intra peritoneal route as the wild type strain. Interestingly, a *S. enterica* serovar Typhi *feoB* mutant exhibited greater virulence defect than the *S. enterica* serovar Typhimurium *tonB* mutant (96). The *S. enterica* serovar Typhi *tonB* mutant was also shown to be attenuated in mice infected intraperitoneally and was defective for growth in HeLa and human monocytes (34, 96).

6.2.3 TonB complex in *Shigella* species

TonB independent iron acquisition is important for the growth of *Shigella flexneri* in the intracellular environment (82, 83). Iron was found to be available to intracellular bacteria, even in the absence of TonB dependent iron transport. The failure of the TonB mutant to grow well in an iron replete intracellular environment suggests that TonB plays a role, in addition to heme and siderophore mediated iron acquisition *in vivo*, and this function is required for intracellular growth and intercellular spread (78). The FeoB of *Shigella dysenteriae* is likely responsible for the acquisition of iron inside the cell (78).

6.2.4 TonB Complex in *Yersinia pestis*

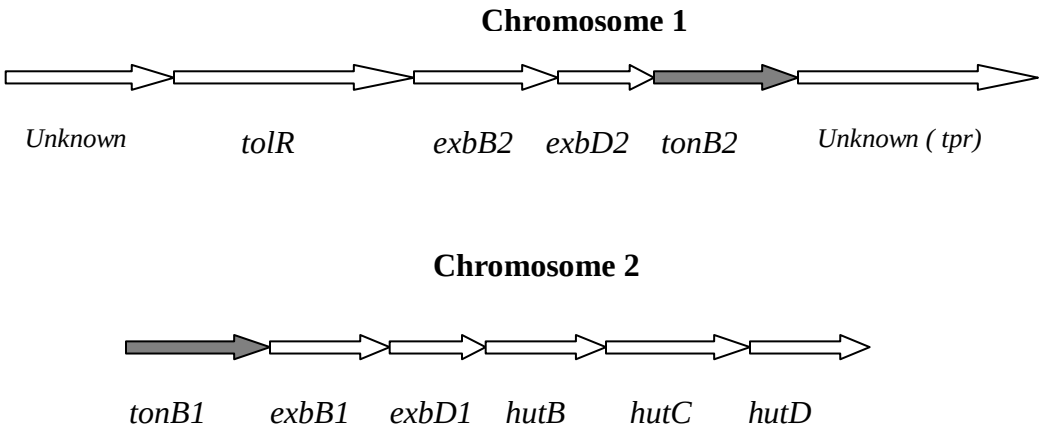
Y. pestis possesses a TonB dependent outer membrane receptor for heme transport and for yersiniabactin mediated iron transport (71, 84). In *Y. enterocolitica*, virulence has been shown to be closely associated with siderophore production (42).

6.3 Multiple TonB Systems

6.3.1 *Vibrio cholerae*

The first identification of multiple TonB genes within the same genome was reported in *V. cholerae* (43, 67). It is a gram-negative human pathogen known to cause cholera and watery diarrhea. *V. cholerae* has two sets of TonBs which confer specific as well as redundant functions (87). The genetic organization of TonBs in *V. cholerae* is shown in Figure 8.

Figure 8: Genetic organization of TonB systems in *Vibrio cholerae*



{Source: Iron Transport in Bacteria (2004) Chapter 16. Vibrio Page 253}

ferrichrome. However, TonB1 is specifically required for the uptake of schizokinen siderophore while TonB2 is essential for enterobactin uptake (62). A double mutant lacking both *tonBs* showed a decreased ability to colonize the intestine of suckling mice, thus demonstrating the importance of the TonB systems for growth in the host (67, 87). The difference in siderophore transport suggests that the two TonB proteins specifically recognize distinct OM transporters. Sequence analysis has shown that TonB1 is bigger than TonB2 by 38 amino acid*s*. This difference enables TonB1 to expand the periplasmic space under conditions of high osmolarity, while the TonB2 is unable to span the increased distance between the CM and OMR at high osmolarity conditions (61). This shows that TonBs have a differential role under different environmental conditions. In *Vibrio anguillarum*, there are two functional TonB systems, TonB1 and TonB2 (93). Each of the TonBs is transcribed in an operon with the cognate ExbB and ExbD in response to iron limitation. However, only TonB2 has been found to be essential for the transport of ferric anguibactin and virulence (93).

6.3.2 *Pseudomonas aeruginosa* TonBs

There exists multiple TonBs, TonB1, TonB2 and PA0695, in *Pseudomonas aeruginosa*, a gram-negative, opportunistic pathogen (108, 110). The *tonB1* gene has been shown to be essential for siderophore mediated iron acquisition (109). Two additional *tonB* genes {*tonB2* and PA0695, (http:/www.pseudomonas.com)} have been identified in this organism, although neither of the two play a significant role in iron uptake. There is also a report about the existence of a fourth *tonB*-like gene in *P. aeruginosa*, *tonB3*, whose product is required for twitching motility (45).

6.3.3 *Actinobacillus pleuropneumoniae* TonBs

A. pleurpneumoniae causes porcine pleuropneumonia, a highly infectious disease of swine (4). There are 2 TonBs: TonB1 and TonB2 in *A. pleuropneumoniae*, and both are upregulated upon iron restriction. TonB2, but not TonB1 was found to be essential for growth *in vitro* when the sole source of iron was hemin, porcine hemoglobin or ferrichrome (4). TonB2 appeared to play a more important role in virulence than TonB1 in an acute porcine infection model (4).

6.3.4 TonBs in *Campylobacter jejuni*

The complete genome sequence of *C. jejuni* NCTC 11168 revealed the presence of three TonB ExbB ExbD complexes (70). The TonB1 protein is 248 amino acids in length and is the largest of the three TonBs in *C. jejuni*. The TonB2 and TonB3 proteins are 227 amino acids each in length and have 41% identity between them. The identity between TonB1 and TonB3 is only 19% and that between TonB1 and TonB2 is 23%. None of the three TonBs are closely situated on the chromosome and their exact role is not yet known. *E. coli* TonB is 22%, 26% and 28% identical to *C. jejuni* TonB1, TonB2 and TonB3 respectively.

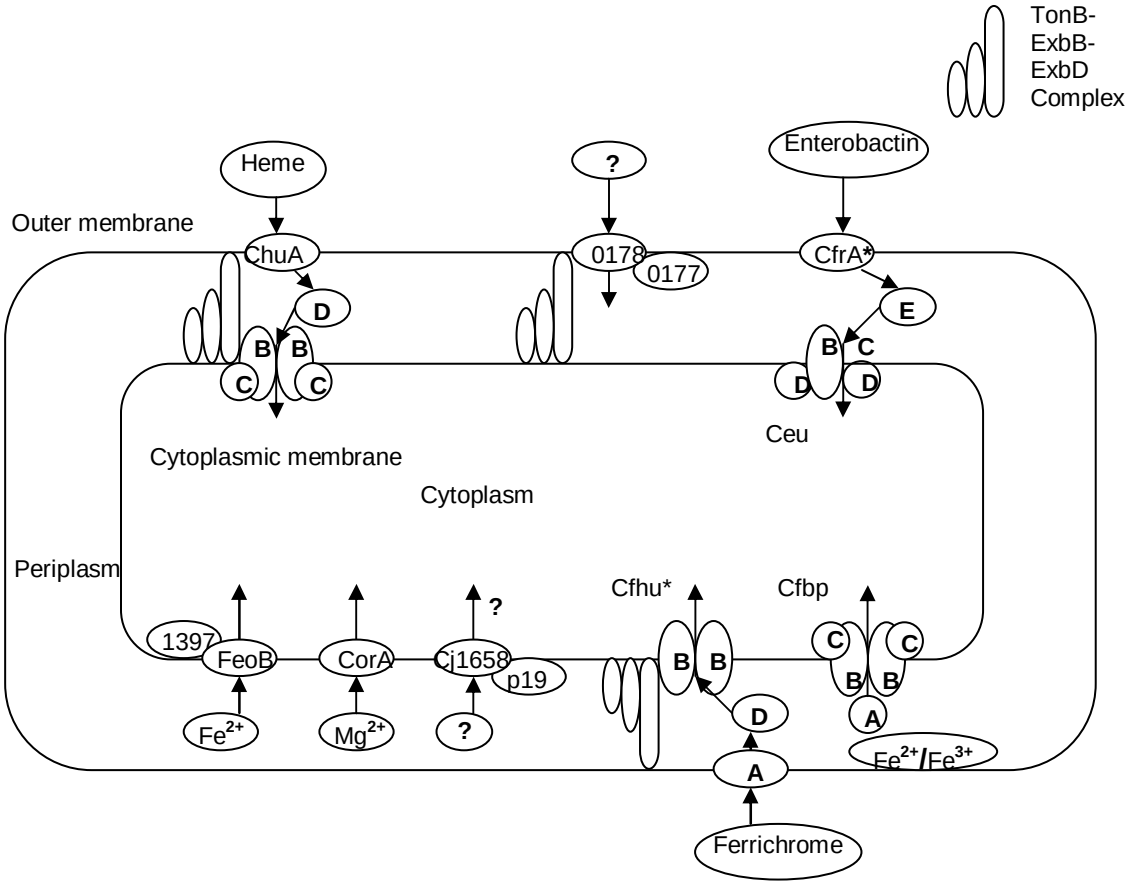
Two of these complexes, ExbB1-ExbD1-TonB1 and ExbB2-ExbD2-TonB2 were recently shown to be up-regulated under iron limited conditions whereas, the ExbB3-ExbD3 wasup-regulated under iron- rich conditions (68). In another recent study, all three TonBs, and ExbB1, ExbB2 , ExbD1 and ExbD2 genes were found to be up-regulated

under iron restricted condition and their relative transcript abundance was ~ three to ten fold increased (44).

7 Ferric Iron acquisition in *C. jejuni*

The *C. jejuni* NCTC 11168 genome sequence analysis has revealed the absence of genes encoding siderophore biosynthesis proteins (70). However, in one study, a few strains have been demonstrated to produce siderophores, and these have been not characterized (29). Bioassays and growth promotion assays with different iron sources have indicated that *C. jejuni* is able to utilize a limited number of iron sources (29). The genome sequence analysis has suggested the presence of several iron uptake systems (70). *C. jejuni* is able to utilize heme compounds like hemin, hemoglobin, enterobactin (29). Ferrichrome uptake system has been identified in some strains of *C. jejuni*; however, the genome of the sequenced strain lacks a ferrichrome uptake system questioning those results (32, 70). *C. jejuni* is unable to utilize aerobactin, desferiferrioxamine, ferritin, lactoferrin and transferrin (29, 72). *C. jejuni* expresses several ferric iron uptake systems as shown in the Figure 9, some of which are partially or completely characterized. These uptake systems have been described in the context of their substrate specificities such as siderophore uptake, heme uptake and unknown substrate specificity(98).

Figure 9: Schematic Model of Iron Uptake Systems in *C. jejuni*



Systems marked with an asterisk (*) are not present in all *C. jejuni* strains.

{Source: Modified from schematic model of iron transport systems of *C. jejuni* A.H.M van Vliet *et al.*, F.E.M.S. Microbiology Reviews 26 (2002) Page 176}

7.1 Enterobactin/Enterochelin uptake

The *ceu* system (Cj1352 - Cj1355) has been identified to encode the *ceuBCDE* operon and is specific for enterobactin uptake (69, 70, 79). CeuE is a lipoprotein and is homologous to siderophore specific periplasmic binding proteins in other gram-negative bacteria. CeuB and CeuC are cytoplasmic permeases and the CeuD is likely an ATPase energizing the transport. The CeuE is annotated as a periplasmic binding protein and putative eneterobactin transporter (98). By growth promotion studies, Palyada *et al*, have shown that the *ceuE* mutant was slightly impaired, although not fully incompetent in its ability to utilize iron from ferric enterobactin-complex (68). However, the *ceuE* mutant was found to be significantly affected in the cecal colonization of chicks (68).

The CfrA protein has been identified as a specific outer membrane receptor for enterobactin uptake in *C. jejuni* NCTC 11168 (68). The *cfrA* mutant was shown to be significantly affected in the chicken cecal colonization studies (68). As *cfrA* is not present in all the *C. jejuni* strains, there maybe other unknown outer membrane receptors for enterobactin uptake in *C. jejuni* (98).

In another recently reported study, Holmes *et al* demonstrated that *ceuE* and *cfrA* transcripts were increased in abundance by 16 fold and 57 fold respectively, under iron limited conditions, thus highlighting the importance of ferric enterobactin utilization in *C. jejuni* (44).

7.2 Ferrichrome uptake

The *C. jejuni* NCTC 11168 genome sequence lacks the presence of a ferrichrome uptake system (70), while a *CFhu ABCD* operon encoding for ferrichrome uptake was identified

in 6 out of 11 *C. jejuni* strains (32). Experimental evidence for ferrichrome uptake is still missing. Nevertheless, an 80 KDa CFhuA protein was identified in *C. jejuni* M129 isolate (32).Therefore, ferrichrome uptake system is not present in all the *C. jejuni* strains and there are no reports about its role in pathogenesis.

7.3 Heme uptake

The *C. jejuni* NCTC11168 genome sequence reveals the presence of a heme uptake system ChuABCD (70). ChuA is highly homologous to the OM siderophore receptors of other bacteria. ChuBCD encode the ABC transporter system (100) . Based on bioassays and mutagenesis studies, there is functional evidence for the role of ChuABCD in *C. jejuni* heme uptake (72). In addition, it has been observed that there was an 87-fold increase in the transcript abundance of ChuA, when *C. jejuni* was grown under iron limited conditions (44). It must be noted that *chuABCD* was only slightly expressed *in vivo*, suggesting that it may not be the main source of iron in the gut (89).

7.4 cFbpA

The *Campylobacter* ferric binding protein (cFbpA) is a periplasmic protein. Recently, its crystal structure was reported (94). The cFbpA iron binding site lacks a synergistic anion, which is responsible for the high affinity for free ferrous iron rather than for free ferric iron(94). The role of cFbpA in *Campylobacter* pathogenesis is not yet established, although recently it has been shown that there was increase in transcript abundance of cFbpA under iron limited conditions (44, 68).

7.5 Cj1658-p19

C. jejuni genome encodes Cj1658-p19 genes which are putative membrane proteins and are potentially involved in iron transport (70). There was an increase in their expression under iron limiting conditions by 37.6 fold and 110 fold for Cj1658 and p19 respectively, while the protein expression level of p19 increased by 11.8 fold (44, 68). They were upregulated *in vivo* when *C. jejuni* lifestyle was studied in the rabbit gut (89). Further functional characterization for studying their role in pathogenesis has not yet been done.

8 Iron and Fur Regulation in *C. jejuni*

8.1 Iron Regulon

The iron regulon of *C. jejuni* has been recently characterized in two independent studies (44, 68). Using DNA microarrays, Palyada *et al.* identified the *C. jejuni* genes that had transcript abundance affected by the availability of iron. They identified 647 genes to be affected as an immediate response after the addition of iron, and only 208 genes were differentially expressed during the steady state experiments where the transcriptome profile was compared under iron rich and iron limiting conditions(44, 68).

Another study (44), Holmes *et al* found an increase in transcript abundance of 117 genes and a decrease in transcript levels of 30 genes in response to iron limitation (44, 68). Genes induced under iron limited conditions encoded for cell surface protein, iron binding and transport systems such as *ChuA*, *cfrA* and *p19*. These genes were also found to be iron regulated in earlier studies (100).

8.2 Fur Regulon

Fur (iron uptake regulator gene) is a global regulator of gene expression in gram-negative bacteria in response to intracellular iron concentration (21), and was identified three decades ago (40). It is a 17 KDa protein (44, 100). Classically, Fur is a transcriptional repressor using Fe(II) as a co-repressor. Homologs of Fur have been found in many gram-negative bacteria such as *Vibrio, Salmonella, Pseudomonas, Helicobacter, Brucella, Yersinia, Legionella, Neisseria, Haemophilus* and others (22).

In *C. jejuni*, the *fur* gene is located in an operon that includes two downstream housekeeping genes *lysS* and *glyA*, leading to a multi-cistronic mRNA (99). The *C. jejuni* Fur protein bears 40% identity with the Fur protein of *E. coli* and is mostly identical at the C-terminal (85). Fur regulated gene have been identified by a Fur titration assay (FURTA) in *E. coli* and *Salmonella* (90, 97) However, FURTA was unsuccessful in identifying genes in *C. jejuni* and *H. pylori* (25). EMSA (Electrophoretic Mobility Shift Assay) was tried recently in *C. jejuni* to compare the *fur* mutant with the wild type Fur protein (44). Also, the mutation of the *fur* gene of *C. jejuni* significantly affected the chicken colonization, indicating that it might play an important role in virulence (68).

The *fur* gene has been demonstrated to repress the transcription of at least seven iron regulated proteins, some of which include CeuE, p19, ChuA, ChuD and CfrA (100). Palyada *et al* recently identified the Fur regulon in *C. jejuni* by transcriptome profiling of the *fur mutant* and the wild type strain. They identified 53 genes to be Fur regulated. The flagellum biogenesis machinery (*FlaD, FlgE2, FlgG2, FlgH and FlgI*), iron acquisition genes (*cfrA, p19, ceuB, ceuC, 1658, tonB, exbB, exbD*) and the oxidative stress defense response gene (*trxB*) were repressed upon addition of iron. They observed six genes to be

up-regulated, including *fdxA* in the presence of iron (68). The Fur regulon was also characterized by Holmes *et al* (44) in another recent study. Their findings were similar to those demonstrated by Palyada *et al*, and they further confirmed the repression of Fur regulated genes in the presence of iron. Under iron limited conditions, the Fur regulated iron acquisition genes and oxidative stress defense response genes were up-regulated (44).

References

1. Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature 397:176-80.

2. Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones. 2003. Bacterial iron homeostasis. FEMS Microbiol Rev 27:215-37.

3. Baichoo, N., and J. D. Helmann. 2002. Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J Bacteriol 184:5826-32.

4. Beddek, A. J., B. J. Sheehan, J. T. Bosse, A. N. Rycroft, J. S. Kroll, and P. R. Langford. 2004. Two TonB systems in *Actinobacillus pleuropneumoniae: t*heir roles in iron acquisition and virulence. Infect Immun 72:701-8.

5. Bister, B., D. Bischoff, G. J. Nicholson, M. Valdebenito, K. Schneider, G. Winkelmann, K. Hantke, and R. D. Sussmuth. 2004. The structure of

salmochelins: C-glucosylated enterobactins of *Salmonella enterica*. Biometals 17:471-81.

6. Bitter, W., J. Tommassen, and P. J. Weisbeek. 1993. Identification and characterization of the *exbB, exbD* and *tonB* genes of *Pseudomonas putida* WCS358: their involvement in ferric-pseudobactin transport. Mol Microbiol 7:117-30.

7. Blanchard, T. G., and S. J. Czinn. 2000. Immunology of *Helicobacter pylori* and prospects for vaccine. Gastroenterol Clin North Am 29:671-85.

8. Blanchard, T. G., M. L. Drakes, and S. J. Czinn. 2004. *Helicobacter* infection: pathogenesis. Curr Opin Gastroenterol 20:10-5.

9. Boyer, E., I. Bergevin, D. Malo, P. Gros, and M. F. Cellier. 2002. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica serovar Typhimurium*. Infect Immun 70:6032-42.

10. Braun, V. 2003. Iron uptake by *Escherichia coli*. Front Biosci 8:s1409-21.

11. Braun, V., and M. Braun. 2002. Active transport of iron and siderophore antibiotics. Curr Opin Microbiol 5:194-201.

12. Braun, V., K. Gunter, and K. Hantke. 1991. Transport of iron across the outer membrane. Biol Met 4:14-22.

13. Brock, J. H., P. H. Williams, J. Liceaga, and K. G. Wooldridge. 1991. Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms. Infect Immun 59:3185-90.

14. Buchanan, S. K., B. S. Smith, L. Venkatramani, D. Xia, L. Esser, M. Palnitkar, R. Chakraborty, D. van der Helm, and J. Deisenhofer. 1999. Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. Nat Struct Biol 6:56-63.

15. Bullen, J. J., H. J. Rogers, P. B. Spalding, and C. G. Ward. 2005. Iron and infection: the heart of the matter. FEMS Immunol Med Microbiol 43:325-30.

16. Cadieux, N., C. Bradbeer, E. Reeger-Schneider, W. Koster, A. K. Mohanty, M. C. Wiener, and R. J. Kadner. 2002. Identification of the periplasmic cobalamin-binding protein BtuF of *Escherichia coli*. J Bacteriol 184:706-17.

17. Carrondo, M. A. 2003. Ferritins, iron uptake and storage from the bacterioferritin viewpoint. Embo J 22:1959-68.

18. Chimento, D. P., A. K. Mohanty, R. J. Kadner, and M. C. Wiener. 2003. Substrate-induced transmembrane signaling in the cobalamin transporter BtuB. Nat Struct Biol 10:394-401.

19. Cornelissen, C. N., and P. F. Sparling. 1994. Identification of receptor-mediated transferrin-iron uptake mechanism in *Neisseria gonorrhoeae*. Methods Enzymol 235:356-63.

20. Cornelissen, C. N., and P. F. Sparling. 1994. Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. Mol Microbiol 14:843-50.

21. Ernst, J. F., R. L. Bennett, and L. I. Rothfield. 1978. Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. J Bacteriol 135:928-34.

22. Escolar, L., J. Perez-Martin, and V. de Lorenzo. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J Bacteriol 181:6223-9.

23. Faraldo-Gomez, J. D., and M. S. Sansom. 2003. Acquisition of siderophores in gram-negative bacteria. Nat Rev Mol Cell Biol 4:105-16.

24. Fassbinder, F., M. Kist, and S. Bereswill. 2000. Structural and functional analysis of the riboflavin synthesis genes encoding GTP cyclohydrolase II (ribA), DHBP

synthase (ribBA), riboflavin synthase (ribC), and riboflavin deaminase/reductase (ribD) from *Helicobacter pylori* strain P1. FEMS Microbiol Lett 191:191-7.

25. Fassbinder, F., A. H. van Vliet, V. Gimmel, J. G. Kusters, M. Kist, and S. Bereswill. 2000. Identification of iron-regulated genes of *Helicobacter pylori* by a modified fur titration assay (FURTA-Hp). FEMS Microbiol Lett 184:225-9.

26. Ferguson, A. D., R. Chakraborty, B. S. Smith, L. Esser, D. van der Helm, and J. Deisenhofer. 2002. Structural basis of gating by the outer membrane transporter FecA. Science 295:1715-9.

27. Ferguson, A. D., and J. Deisenhofer. 2004. Metal import through microbial membranes. Cell 116:15-24.

28. Ferguson, A. D., E. Hofmann, J. W. Coulton, K. Diederichs, and W. Welte. 1998. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science 282:2215-20.

29. Field, L. H., V. L. Headley, S. M. Payne, and L. J. Berry. 1986. Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. Infect Immun 54:126-32.

30. Furrer, J. L., D. N. Sanders, I. G. Hook-Barnard, and M. A. McIntosh. 2002. Export of the siderophore enterobactin in *Escherichia coli:* involvement of a 43 kDa membrane exporter. Mol Microbiol 44:1225-34.

31. Gaisser, S., and V. Braun. 1991. The *tonB* gene of *Serratia marcescens*: sequence, activity and partial complementation of Escherichia coli tonB mutants. Mol Microbiol 5:2777-87.

32. Galindo, M. A., W. A. Day, B. H. Raphael, and L. A. Joens. 2001. Cloning and characterization of a *Campylobacter jejuni* iron-uptake operon. Curr Microbiol 42:139-43.

33. Ghosh, A., M. Ghosh, C. Niu, F. Malouin, U. Moellmann, and M. J. Miller. 1996. Iron transport-mediated drug delivery using mixed-ligand siderophore-beta-lactam conjugates. Chem Biol 3:1011-9.

34. Gorbacheva, V. Y., G. Faundez, H. P. Godfrey, and F. C. Cabello. 2001. Restricted growth of ent(-) and tonB mutants of *Salmonella enterica serovar Typhi* in human Mono Mac 6 monocytic cells. FEMS Microbiol Lett 196:7-11.

35. Guerinot, M. L. 1994. Microbial iron transport. Annu Rev Microbiol 48:743-72.

36. Hantash, F. M., M. Ammerlaan, and C. F. Earhart. 1997. Enterobactin synthase polypeptides of *Escherichia coli* are present in an osmotic-shock-sensitive cytoplasmic locality. Microbiology 143 (Pt 1):147-56.

37. Hantash, F. M., and C. F. Earhart. 2000. Membrane association of the *Escherichia coli* enterobactin synthase proteins EntB/G, EntE, and EntF. J Bacteriol 182:1768-73.

38. Hantke, K. 1997. Ferrous iron uptake by a magnesium transport system is toxic for *Escherichia coli* and *Salmonella typhimurium*. J Bacteriol 179:6201-4.

39. Hantke, K. 2003. Is the bacterial ferrous iron transporter FeoB a living fossil? Trends Microbiol 11:192-5.

40. Hantke, K. 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol Gen Genet 182:288-92.

41. Hantke, K., G. Nicholson, W. Rabsch, and G. Winkelmann. 2003. Salmochelins, siderophores of *Salmonella enterica* and uropathogenic *Escherichia coli* strains, are recognized by the outer membrane receptor IroN. Proc Natl Acad Sci U S A 100:3677-82.

42. Heesemann, J., K. Hantke, T. Vocke, E. Saken, A. Rakin, I. Stojiljkovic, and R. Berner. 1993. Virulence of *Yersinia enterocolitica* is closely associated with siderophore production, expression of an iron-repressible outer membrane polypeptide of 65,000 Da and pesticin sensitivity. Mol Microbiol 8:397-408.

43. Henderson, D. P., and S. M. Payne. 1994. *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. Infect Immun 62:5120-5.

44. Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells. 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology 151:243-57.

45. Huang, B., K. Ru, Z. Yuan, C. B. Whitchurch, and J. S. Mattick. 2004. *tonB3* is required for normal twitching motility and extracellular assembly of type IV pili. J Bacteriol 186:4387-9.

46. Ingelman, M., S. Ramaswamy, V. Niviere, M. Fontecave, and H. Eklund. 1999. Crystal structure of NAD(P)H:flavin oxidoreductase from *Escherichia coli*. Biochemistry 38:7040-9.

47. Janakiraman, A., and J. M. Slauch. 2000. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Mol Microbiol 35:1146-55.

48. Jeffrey, P. D., M. C. Bewley, R. T. MacGillivray, A. B. Mason, R. C. Woodworth, and E. N. Baker. 1998. Ligand-induced conformational change in transferrins: crystal structure of the open form of the N-terminal half-molecule of human transferrin. Biochemistry 37:13978-86.

49. Kammler, M., C. Schon, and K. Hantke. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J Bacteriol 175:6212-9.

50. Kelly, C., and V. Saravanan. 2003. Helicobacter pylori infection in rheumatoid arthritis: effect of drugs on prevalence and correlation with gastroduodenal lesions. Rheumatology (Oxford) 42:388; author reply 388.

51. Kelly, D. J. 2001. The physiology and metabolism of *Campylobacter jejuni* and *Helicobacter pylori*. Symp Ser Soc Appl Microbiol:16S-24S.

52. Kelly, D. J. 1998. The physiology and metabolism of the human gastric pathogen *Helicobacter pylori*. Adv Microb Physiol 40:137-89.

53. Kelly, S. M., J. M. Geraghty, and G. Neale. 1994. H pylori, gastric carcinoma, and MALT lymphoma. Lancet 343:418.

54. Kingsley, R. A., R. Reissbrodt, W. Rabsch, J. M. Ketley, R. M. Tsolis, P. Everest, G. Dougan, A. J. Baumler, M. Roberts, and P. H. Williams. 1999. Ferrioxamine-mediated Iron(III) utilization by *Salmonella enterica*. Appl Environ Microbiol 65:1610-8.

55. Komai, H., and J. B. Neilands. 1969. The metalloprotein nature of *Ustilago* delta-aminolevulinate dehydratase. Biochim Biophys Acta 171:311-20.

56. Kurokawa, H., J. C. Dewan, B. Mikami, J. C. Sacchettini, and M. Hirose. 1999. Crystal structure of hen apo-ovotransferrin. Both lobes adopt an open conformation upon loss of iron. J Biol Chem 274:28445-52.

57. Larsen, R. A., P. S. Myers, J. T. Skare, C. L. Seachord, R. P. Darveau, and K. Postle. 1996. Identification of TonB homologs in the family Enterobacteriaceae and evidence for conservation of TonB-dependent energy transduction complexes. J Bacteriol 178:1363-73.

58. Larsen, R. A., M. G. Thomas, and K. Postle. 1999. Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol Microbiol 31:1809-24.

59. Locher, K. P., B. Rees, R. Koebnik, A. Mitschler, L. Moulinier, J. P. Rosenbusch, and D. Moras. 1998. Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell 95:771-8.

60. Marlovits, T. C., W. Haase, C. Herrmann, S. G. Aller, and V. M. Unger. 2002. The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. Proc Natl Acad Sci U S A 99:16243-8.

61. Mey, A. R., and S. M. Payne. 2003. Analysis of residues determining specificity of *Vibrio cholerae* TonB1 for its receptors. J Bacteriol 185:1195-207.

62. Mey, A. R., and S. M. Payne. 2001. Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. Mol Microbiol 42:835-49.

63. Moeck, G. S., and J. W. Coulton. 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol Microbiol 28:675-81.

64. Neilands, J. B. 1981. Iron absorption and transport in microorganisms. Annu Rev Nutr 1:27-46.

65. Neilands, J. B. 1993. Siderophores. Arch Biochem Biophys 302:1-3.

66. Neilands, J. B. 1995. Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 270:26723-6.

67. Occhino, D. A., E. E. Wyckoff, D. P. Henderson, T. J. Wrona, and S. M. Payne. 1998. *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB, exbB, exbD* genes. Mol Microbiol 29:1493-507.

68. Palyada, K., D. Threadgill, and A. Stintzi. 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol 186:4714-29.

69. Park, S. F., and P. T. Richardson. 1995. Molecular characterization of a *Campylobacter jejuni* lipoprotein with homology to periplasmic siderophore-binding proteins. J Bacteriol 177:2259-64.

70. Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-8.

71. Perry, R. D., J. Shah, S. W. Bearden, J. M. Thompson, and J. D. Fetherston. 2003. *Yersinia pestis* TonB: role in iron, heme, and hemoprotein utilization. Infect Immun 71:4159-62.

72. Pickett, C. L., T. Auffenberg, E. C. Pesci, V. L. Sheen, and S. S. Jusuf. 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. Infect Immun 60:3872-7.

73. Ponka, P., C. Beaumont, and D. R. Richardson. 1998. Function and regulation of transferrin and ferritin. Semin Hematol 35:35-54.

74. Posey, J. E., and F. C. Gherardini. 2000. Lack of a role for iron in the Lyme disease pathogen. Science 288:1651-3.

75. Postle, K., and R. J. Kadner. 2003. Touch and go: tying TonB to transport. Mol Microbiol 49:869-82.

76. Raphael, B. H., and L. A. Joens. 2003. FeoB is not required for ferrous iron uptake in *Campylobacter jejuni.* Can J Microbiol 49:727-31.

77. Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol 54:881-941.

78. Reeves, S. A., A. G. Torres, and S. M. Payne. 2000. TonB is required for intracellular growth and virulence of *Shigella dysenteriae*. Infect Immun 68:6329-36.

79. Richardson, P. T., and S. F. Park. 1995. Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system. Microbiology 141 (Pt 12):3181-91.

80. Robson, H. G., and S. I. Vas. 1972. Resistance of inbred mice to *Salmonella typhimurium.* J Infect Dis 126:378-86.

81. Rossi, M. S., A. Paquelin, J. M. Ghigo, and C. Wandersman. 2003. Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens:* the inducer and the transported substrate are different molecules. Mol Microbiol 48:1467-80.

82. Runyen-Janecky, L. J., and S. M. Payne. 2002. Identification of chromosomal *Shigella flexneri* genes induced by the eukaryotic intracellular environment. Infect Immun 70:4379-88.

83. Runyen-Janecky, L. J., S. A. Reeves, E. G. Gonzales, and S. M. Payne. 2003. Contribution of the *Shigella flexneri* Sit, Iuc, and Feo iron acquisition systems to iron acquisition in vitro and in cultured cells. Infect Immun 71:1919-28.

84. Saken, E., A. Rakin, and J. Heesemann. 2000. Molecular characterization of a novel siderophore-independent iron transport system in *Yersinia*. Int J Med Microbiol 290:51-60.

85. Schaffer, S., K. Hantke, and V. Braun. 1985. Nucleotide sequence of the iron regulatory gene fur. Mol Gen Genet 200:110-3.

86. Schalk, I. J., P. Kyslik, D. Prome, A. van Dorsselaer, K. Poole, M. A. Abdallah, and F. Pattus. 1999. Copurification of the FpvA ferric pyoverdin receptor of *Pseudomonas aeruginosa* with its iron-free ligand: implications for siderophore-mediated iron transport. Biochemistry 38:9357-65.

87. Seliger, S. S., A. R. Mey, A. M. Valle, and S. M. Payne. 2001. The two TonB systems of *Vibrio cholerae*: redundant and specific functions. Mol Microbiol 39:801-12.

88. Stintzi, A., C. Barnes, J. Xu, and K. N. Raymond. 2000. Microbial iron transport via a siderophore shuttle: a membrane ion transport paradigm. Proc Natl Acad Sci U S A 97:10691-6.

89. Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke. 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect Immun 73:1797-810.

90. Stojiljkovic, I., A. J. Baumler, and K. Hantke. 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay. J Mol Biol 236:531-45.

91. Stojiljkovic, I., M. Cobeljic, and K. Hantke. 1993. *Escherichia coli* K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. FEMS Microbiol Lett 108:111-5.

92. Stokes, R. H., J. S. Oakhill, C. L. Joannou, A. R. Gorringe, and R. W. Evans. 2005. Meningococcal transferrin-binding proteins A and B show cooperation in their binding kinetics for human transferrin. Infect Immun 73:944-52.

93. Stork, M., M. Di Lorenzo, S. Mourino, C. R. Osorio, M. L. Lemos, and J. H. Crosa. 2004. Two tonB systems function in iron transport in *Vibrio anguillarum*, but only one is essential for virulence. Infect Immun 72:7326-9.

94. Tom-Yew, S. A., D. T. Cui, E. G. Bekker, and M. E. Murphy. 2004. Anion-independent iron coordination by the *Campylobacter jejun*i ferric binding protein. J Biol Chem.

95. Torres, A. G., P. Redford, R. A. Welch, and S. M. Payne. 2001. TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. Infect Immun 69:6179-85.

96. Tsolis, R. M., A. J. Baumler, F. Heffron, and I. Stojiljkovic. 1996. Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. Infect Immun 64:4549-56.

97. Tsolis, R. M., A. J. Baumler, I. Stojiljkovic, and F. Heffron. 1995. Fur regulon of *Salmonella typhimurium*: identification of new iron-regulated genes. J Bacteriol 177:4628-37.

98. van Vliet, A. H., J. M. Ketley, S. F. Park, and C. W. Penn. 2002. The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. FEMS Microbiol Rev 26:173-86.

99. van Vliet, A. H., J. D. Rock, L. N. Madeleine, and J. M. Ketley. 2000. The iron-responsive regulator Fur of *Campylobacter jejuni* is expressed from two separate promoters. FEMS Microbiol Lett 188:115-8.

100. van Vliet, A. H., K. G. Wooldridge, and J. M. Ketley. 1998. Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. J Bacteriol 180:5291-8.

101. Velayudhan, J., N. J. Hughes, A. A. McColm, J. Bagshaw, C. L. Clayton, S. C. Andrews, and D. J. Kelly. 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. Mol Microbiol 37:274-86.

102. Wandersman, C., and P. Delepelaire. 2004. Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol 58:611-47.

103. Way, S. S., and M. B. Goldberg. 1998. Clearance of *Shigella flexneri* infection occurs through a nitric oxide-independent mechanism. Infect Immun 66:3012-6.

104. Wei, J., M. B. Goldberg, V. Burland, M. M. Venkatesan, W. Deng, G. Fournier, G. F. Mayhew, G. Plunkett, 3rd, D. J. Rose, A. Darling, B. Mau, N. T. Perna, S. M. Payne, L. J. Runyen-Janecky, S. Zhou, D. C. Schwartz, and F. R. Blattner. 2003. Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect Immun 71:2775-86.

105. Worst, D. J., M. M. Gerrits, C. M. Vandenbroucke-Grauls, and J. G. Kusters. 1998. *Helicobacter pylori* ribBA-mediated riboflavin production is involved in iron acquisition. J Bacteriol 180:1473-9.

106. Wu, R., E. P. Skaar, R. Zhang, G. Joachimiak, P. Gornicki, O. Schneewind, and A. Joachimiak. 2005. *Staphylococcus aureus* IsdG and IsdI, heme-degrading enzymes with structural similarity to monooxygenases. J Biol Chem 280:2840-6.

107. Yue, W. W., S. Grizot, and S. K. Buchanan. 2003. Structural evidence for iron-free citrate and ferric citrate binding to the TonB-dependent outer membrane transporter FecA. J Mol Biol 332:353-68.

108. Zhao, Q., X. Z. Li, A. Mistry, R. Srikumar, L. Zhang, O. Lomovskaya, and K. Poole. 1998. Influence of the TonB energy-coupling protein on efflux-mediated multidrug resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 42:2225-31.

109. Zhao, Q., and K. Poole. 2002. Mutational analysis of the TonB1 energy coupler of *Pseudomonas aeruginosa*. J Bacteriol 184:1503-13.

110. Zhao, Q., and K. Poole. 2000. A second tonB gene in *Pseudomonas aeruginosa* is linked to the *exbB* and *exbD* genes. FEMS Microbiol Lett 184:127-32.

Chapter III

Contribution Of The *Campylobacter Jejuni* FeoB Iron Acquisition System For *In Vivo* Colonization And *In Vitro* Growth

Hemant Naikare, Kiran Palyada and Alain Stintzi

Department of Veterinary Pathobiology

Oklahoma State University

Stillwater OK 74078

Abstract

Fe (II) uptake via the cytoplasmic membrane protein FeoB has been well characterized in many bacterial enteric pathogens and has been shown to significantly contribute towards iron acquisition and the establishment of infection. The genome sequence of *Campylobacter jejuni* reveals the presence of a Fe (II) transporter, FeoB. $^{55}Fe^{2+}$ uptake via FeoB was abolished by the membrane protonophore carboxyl cyanide-m-chlorphenyl hydrazone (CCCP), indicating proton dependency for ferrous iron transport. Using an allelic exchange strategy, we constructed mutants of *feoB* gene in three strains of *C. jejuni*, *C. jejuni* NCTC 11168 and two clinical isolates, *C. jejuni* ATCC 43431 (TGH 9011) and *C. jejuni* 81-176. The *feoB* mutant had a significantly lower cellular iron accumulation than the wild type strain and was significantly affected in the $^{55}Fe^{2+}$ uptake. Microarray experiments identified 110 genes to be differentially expressed between the *feoB* mutant and wild-type *C. jejuni* NCTC 11168. Interestingly, it revealed the upregulation of the oxidative stress defense gene *katA* and the gene encoding the non-heme iron protein Cj0012c in the *feoB* mutant strain. Furthermore, the *feoB* mutant was found to be significantly affected in its ability to survive within porcine (IPEC-1) and human (INT-407) intestinal cell lines. A significant extracellular ferric reductase activity was identified from both the wild type strain *C. jejuni* NCTC 11168 and the *feoB* mutant. Finally, the *feoB* mutants were extensively assessed *in vivo* for their ability to colonize the gastrointestinal tract of chicks, colostrum-deprived neonatal piglets and rabbits. Interestingly, the *feoB* mutants were significantly affected in the gut colonization of all the animal models screened by competitive and non-competitive colonization assays. This is the first study documenting the use of neonatal piglets in screening mutants of

C. jejuni and use of IPEC-1 porcine epithelial cells to assess the intracellular survival of *C. jejuni* wild type and *feoB* mutants. Overall, this work indicates that FeoB significantly contributes towards iron acquisition and in the colonization of *C. jejuni* within the animal host.

Introduction

Campylobacter jejuni is the most common cause of human enteritis worldwide. Contamination of poultry products with gut contents during slaughter is a major source of *C. jejuni* infection. The signs and symptoms associated with campylobacteriosis vary from mild non-inflammatory diarrhea to severe dysentery (6, 13, 29). The complete genome sequence of *C. jejuni* NCTC 11168 was released in 2000 (18). It has been an important tool for the post-genomic investigations in understanding the physiology of *C. jejuni* and deciphering the molecular mechanisms of its pathogenesis. Potential virulence factors include chemotaxis, motility, adhesion, invasion, intracellular survival, toxin production and iron acquisition (13, 29, 32, 33). The importance of iron acquisition for the successful colonization of the host has been demonstrated for many pathogens (23). The significance of iron acquisition and regulation in *C. jejuni* has been recently reported and is based on the global gene expression studies in response to iron (10, 17). Also, the role of iron in *C. jejuni* colonization *in vivo* using the chick colonization model has been previously reported (17). Furthermore, the intestinal lifestyle of *C. jejuni* in the rabbit gut has been recently studied, and it suggests an important role for iron acquisition genes during colonization (26).

Analysis of the *C. jejuni* NCTC 11168 genome reveals the presence of a *feoB* homolog (Cj1398) which is about 1.8 Kb in size and encodes a 70 KDa cytoplasmic membrane protein (18). *C. jejuni* FeoB shares 50% and 29% amino acid identity with *Helicobacter pylori* and *Escherichia coli*, respectively. Cj1398 appears to be in an operonic structure

with Cj1397. Cj1397 encodes a putative protein of 74 amino acids and bears 16% identity to the *E. coli* FeoA protein. However, its role in iron transport is not yet known (29).

The FeoB protein is highly conserved in many species of gram-negative bacteria (8). The FeoB protein contains a G-protein domain suggesting that FeoB energizes the transport of ferrous iron through the cytoplasmic membrane via GTP hydrolysis (8, 15). The *feoAB* operon of *E. coli* is the first well characterized Fe (II) iron transport system (12). Fe (II) iron transport through the membrane protein FeoB has been shown to be required for infectivity and host tissue colonization by *E. coli*, V. *cholerae* and other enteric pathogens (12, 30). In contrast to all other characterized FeoB transporter systems in bacteria, the FeoB protein from *C. jejuni* has been recently proposed to be not essential for Fe(II) iron uptake (22).

In the present study, we re-investigated the role of FeoB in iron acquisition in *C. jejuni* and tested its requirement for the colonization/infectivity of *C. jejuni* in animal hosts. To achieve this purpose, we constructed *C. jejuni feoB* mutants in the wild type strain *C. jejuni* NCTC 11168 and two clinical isolates, *C. jejuni* 81-176 and *C. jejuni* ATCC 43431 (TGH 9011) by allelic exchange, and characterized them *in vitro* and *in vivo.*

Materials and Methods

Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α was routinely cultured aerobically at 37˚C in Luria-Bertani (LB) broth or on LB agar plates. Plasmid containing strains were grown in a medium supplemented with kanamycin at 30 μg/ml. *C. jejuni* NCTC 11168 was acquired from the National Collection of Type Cultures, whereas *C. jejuni* 81-176 and *C. jejuni* ATCC 43431 (TGH 9011) were acquired from Dr Pickett and the American Type Culture Collection, respectively. These strains were routinely grown at 37˚C in a MACS-VA500 microaerophilic work station (Don Whitley, West Yorkshire, England) under 83% N_2, 4% H_2, 8% O_2 and 5% CO_2 on Mueller Hinton (MH) agar plates, MH broth medium or minimum essential medium (MEMα, Invitrogen). Kanamycin was added as required at a concentration of 30 μg/ml. Prior to performing any *in vitro* cell culture studies or *in vivo* animal experiments, the *C. jejuni* strains were checked for motility on 0.4% MH agar plates.

Cell Culture

Human INT-407 embryonic intestinal cells were obtained from the American Type Culture Collection and routinely maintained in minimum essential medium (MEMα, Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen). Porcine IPEC-1 small intestinal epithelial cells were obtained from Dr Zhang (Animal Science Department, OSU) and were routinely maintained on Dulbecco's minimum essential

medium (DMEM, Invitrogen), supplemented with 5% fetal bovine serum (Invitrogen), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (ITS, Invitrogen), and epidermal growth factor (5 ng/ml), (EGF, Invitrogen). Cells were grown in an incubator at 37˚C under 5% CO_2.

Bacterial binding, invasion and intracellular survival assays

These assays were performed as described previously (21). In brief, the binding assay was performed by co-incubation of *C. jejuni* cells grown to mid-log-phase in biphasic MH media with 24 hour grown, semi-confluent INT-407 cells, or 48 hour grown IPEC-1 cells (about 10^5 cells per well) at a multiplicity of infection (MOI) of 10:1 (10 bacteria per eukaryotic cell). After three hours of incubation at 37˚C in the presence of 5 % CO_2, the cell growth medium was removed, and the monolayer was washed thrice with Hanks balanced salt solution (HBBS). Thereafter, the infected cells were lysed with 0.1 % Triton X-100 at room temperature for 30 minutes. Serial dilutions of the cell lysates were plated on MH agar plates to enumerate the number of bacteria bound to and internalized within the eukaryotic cells.

A similar procedure was followed for the invasion assay, with the exception of a further incubation of the infected cells for an additional hour in fresh medium with 250 μg of gentamicin per ml, to kill the extracellular bacteria. Thereafter, the cells were washed thrice with Hanks balanced salt solution, and lysed using 0.1 % Triton X-100 for 30 minutes at room temperature. Serial dilutions of cell lysates were plated on MH agar plates in order to enumerate the number of bacteria invaded within the eukaryotic cells.

The binding efficiency was obtained by subtracting the number of invaded bacteria from the total number of bacteria recovered from cells not subjected to gentamicin treatment, whereas the invasion efficiency was expressed as the percentage of inoculum recovered after gentamicin treatment. The results shown for the binding and invasion assays represent the means of three independent experiments ± standard error.

The intracellular survival (ICS) assay was performed with semi-confluent INT-407 and IPEC-1 intestinal epithelial cells, similar to the invasion assay as described above. After incubation with gentamicin for the killing of extracellular bacteria, the cells were washed thrice using HBSS and were cultured for an additional 24, 48 and 72 hours in fresh medium without antibiotics. The medium was changed every 24 hours. After the desired incubation time points, the monolayer were washed three times with HBSS, and then lysed with 0.1 % Triton X-100 at room temperature for 30 minutes. The number of viable intracellular bacteria was determined by plating the serially diluted cell lysate suspensions on MH agar plates. The experiment was repeated thrice, and the results shown are the mean ± standard error.

Construction of *C. jejuni* NCTC microarray

The microarray was constructed as previously described (25, 26) using DNA fragments covering approximately 98% of all of the open reading frames (ORFs) from *C. jejuni* NCTC 11168.

Total RNA extraction

The iron restricted wild type or mutant *feoB* cells were grown in 100 ml of MEM α under microaerophilic conditions. At the mid-log-phase, (OD_{600} ≈ 0.2), 15 ml of the samples were removed and immediately mixed with 1.5 ml cold RNA degradation stop solution (10% phenol in ethanol). The cells were pelleted by centrifugation at 4˚C for 10 minutes at 8000 X g and then resuspended in lysozyme-TE buffer [50 mM Tris-Cl (pH 8), 1 mM EDTA, 0.5 mg/ml lysozyme]. Total RNA was extracted using a hot phenol-chloroform procedure, as previously described (17). Total RNA was suspended in RNase-free water and subjected to two successive DNase-1 (Invitrogen) treatments to remove any contaminating chromosomal DNA. Finally, RNA was further purified using the RNeasy kit from Qiagen. The absence of genomic DNA was ensured by PCR using several of the primers designed for the microarray construction. The RNA integrity was confirmed by agarose gel electrophoresis, followed by quantification with RiboGreen RNA quantification reagent (Molecular probes). This RNA was then stored at -80˚C until further use.

Labeling of probes and hybridization on slides

The total RNA from each growth condition was converted to cDNA with Superscript II enzyme (Invitrogen), in the presence of aminoallyl-dUTP at 42˚C as previously described. The protocol for the reverse transcription reaction was as follows: 16 μg of total RNA was mixed with 10 μg of random hexamers in a 34.35 μl reaction mixture, containing 2 μl of 0.1 M dithiothreitol and 8 μl of 5 X Superscript II reverse transcriptase

buffer. The reaction mixture was made up to a final volume of 40 μl after a 5 minute incubation at 65˚C, by the addition of 0.5 mM dGTP, 0.5 mM ATP, 0.5 mM CTP, 0.34 mM amino allyl-dUTP, 0.16 mM dTTP and 2 μl of Superscript II (200 units/μl) and incubated at 42˚C for 120 minutes. Then the reverse transcription was stopped and the RNA was hydrolyzed by the addition of 2 μl of 10 N NaOH and 4 μl of 50 mM EDTA, and incubation at 65 ˚C for 20 minutes. This reaction was neutralized by adding 4 μl of 5 M acetic acid. Separation of aminoallyl-labeled cDNA from unincorporated aminoallyl-labeled-dUTP and free amines was achieved by the addition of 450 μl of water, followed by spinning through a Microcon YM-30 filter (Millipore) at 8000 X g for 8 minutes. This step was repeated thrice. Following the last wash, the aminoallyl-labeled probes were subjected to vacuum in a Speed Vac to condense the volume to less than 9 μl, after which, a final volume of 10 μl was attained by the addition of 1 μl of 1 M sodium carbonate (pH 9.0) and water. The aminoallyl-labeled cDNA thus obtained was linked to monoreactive fluors (Amersham), by the addition of 10 μl dimethyl sulfoxide containing one-sixth of one reaction vial of FluoroLink indodicarbocyanine or indocarbocyanine dye, and was incubated for 45 minutes in the dark, at room temperature. Quenching of this reaction was achieved by the addition of 4.5 μl of 4 M hydroxylamine, followed by incubation at room temperature for 15 minutes, in the dark. The fluorescent indodicarbocyanine- and indocarbocyanine-labeled cDNAs were combined and purified using Qiaquick PCR spin columns (Qiagen, Valencia, Calif.). The fluor-labeled cDNA mixture was vacuum dried with a Speed Vac prior to resuspension in 15.14 μl of water. To this resuspension was added 9 μl of 20 X SSC (1 X SSC is 0.15 M NaCl + 0.015 M sodium citrate pH 7.0), 9 μl of formamide, 0.36 μl of 10 % SDS and 2.5

μl of salmon sperm DNA (10 mg/ml). The microarray slides were prehybridized at 42 ˚C for 45 minutes in a prehybridization buffer containing 25 % formamide, 5 X SSC buffer, 1 % bovine serum albumin and 0.1 % SDS. Prior to hybridization, the slides were rinsed with water and dried by spinning. Denaturation of the probe was achieved by boiling for two minutes, followed by cooling to 42˚C. The probe was applied to the microarray slide under a coverslip (Grace Bio-labs) and placed in a humidified chamber (ArrayIt), followed by overnight incubation at 42˚C. Post hybridization, the slides were washed for 5 minutes in 2 X SSC, 0.1 % SDS at 42˚C, 10 minutes in 0.1 X SSC-0.1 % SDS at room temperature, and then four times in 0.1 X SSC for one minute at room temperature. The slides were subsequently rinsed with distilled water and dried by centrifugation.

Data collection and analysis

The microarray slides were scanned at 10 μm resolution using a Scan Array 5000 confocal scanner (Perkin Elmer) and analyzed with GenePix Pro 4.0 software (Axon Instruments, Foster City, Calif.). Spots were excluded from further analysis if either of the following two criteria were met: 1) the mean intensities of fluorescence were less than 3 times the standard deviation of the local background in both channels 1 (indodicarbocyanine) and 2 (indocarbocyanine); 2) the spots were contained in regions of slide hybridizations or anomalies.

Normalization of the fluorescent intensities in each wavelength was achieved by applying a locally weighted linear regression (Lowess) using the MIDAS software (available from TIGR, http://www.tigr.org/software/). After normalization, the ratio of channels 2 to 1

was transformed to $\log_2$, following which, the data were statistically analyzed using a Student *t* test. The experiment was repeated three times (3 biological replicates). Three measurements per gene were obtained per experiment and thus, there were nine measurements for every gene.

Construction of the *C. jejuni feoB* mutants by allelic exchange

C. jejuni NCTC 11168 chromosomal DNA was extracted using the Wizard genomic DNA purification kit (Promega). The *feoB* gene was PCR amplified with the primers *feo-1* (5'CGCTGGCCAAAGTCATTGATTTGCCAGGAAC3') and *feo-2* (5'CGCTGGCCAGCCACTGCACTTGGTATAGG3') (both containing a *Mlu*NI restriction site). These primers annealed within the *feoB* gene and amplified a fragment of 1617 bp. The resultant fragment was digested with *Mlu*NI and ligated to the *Mlu*NI-restricted pCAP vector to obtain pAS43 (pCAP-*feoB*). Then, the *feoB* gene was disrupted by cloning in its unique *Cla*I site a *Cla*I-restricted kanamycin resistant cassette obtained from pILL600, yielding pAS223 (pCAP::*feoB::Km*). The orientation of the Km^r cassette was determined to be in the same direction as the *feoB* gene by PCR and restriction digestion. This final construct pAS223 was used to transform *C. jejuni* NCTC11168 using previously established protocols (36). The *feoB* mutants were selected on MH agar plates containing 30 μg of kanamycin per ml, and the homologous recombination event was confirmed by PCR using a combination of primers that amplified the Km^r and *feoB* genes. The primers and plasmids used for the mutant construction are listed in Table 2. The same plasmid pAS223 was used to construct the *feoB* mutants in the non-sequenced *C. jejuni* 81-176 and *C. jejuni* TGH 9011.

Cj1397-Cj1398 operon mapping

Total RNA was purified as described above, from mid-log-phase *C. jejuni* grown in MEMα medium. First strand cDNA synthesis and subsequent PCR were performed using the Qiagen one step RT-PCR kit following the manufacturer's recommendations. 100 ng of total RNA was used for each reaction. The primers used in the RT-PCR reaction are listed in Table 2. In order to confirm the absence of contaminating genomic DNA, PCR reactions (without previous reverse transcription) were performed using the same RNA templates, as a negative control. The RT-PCR products were electrophoresed in 0.9% agarose gel. 100 bp and 1 Kb standard ladder (Bayou-BioLabs) were run simultaneously, as can be seen in Figure 12.

$^{55}Fe^{2+}$ uptake assays

The $^{55}Fe^{2+}$ uptake assays were performed with the *feoB* mutant and *C. jejuni* wild-type cells grown to mid-log-phase in MH, MH-desferriferrioxamine (20 μM) and iron limited MEMα media. The cells were pelleted and washed in 10 mM Tris buffer (pH 7.4). Then, the cells were resuspended in the uptake buffer to an OD_{600}=0.6 (equivalent to 10^9 bacteria/ml) and kept on ice. The uptake buffer was composed of 5 g/L Na_2HPO_4, 5 g/L KH_2PO_4, 1.18 g/L NH_4Cl, 0.089 g/L Na_2SO_4, 0.042 g/L $MgCl_2.6H_2O$ and 10 g/L casamino acids. Then, the resuspended cells were allowed to equilibrate at 37˚C for 10 minutes before initiating the assay, by adding $^{55}Fe^{2+}$ iron source in the reduced form. The $^{55}Fe^{2+}$ iron source was prepared by diluting the stock $^{55}FeCl_3$, using 1 M sodium

ascorbate as follows: 1µl of 3.7 mM stock $^{55}FeCl_3$ was diluted with 60 µl of 1M sodium ascorbate and 940 µl of the uptake buffer to maintain the radiolabeled iron in the reduced Fe(II) state. 100 µl of the diluted Fe(II) iron source was added to 10 ml of the resuspended cells, providing a final concentration of 0.036 µM, and the ferrous iron uptake was initiated. Samples (1 ml) were taken every 2 minutes and the cells were pelleted by centrifugation at 13000 rpm for 2 minutes. The pelleted cells were washed twice using 0.1 M citric acid buffer and finally resuspended in 500 µl of cold water. This suspension was placed in 5 ml of scintillation cocktail (Scintiverse, Fisher) and counted in Beckman LS5000 TD scintillation counter. Activity as counts per minute (CPM) were corrected for background. For proton dependency studies, cells were treated with 33 µM of proton ionophore carbonyl-cyanide-m-chlorphenyl hydrazone (CCCP) prior to the addition of the labeled ferrous iron with another batch of cells.

Subcellular fractionation

For confirmation of the actual ferrous iron uptake from the periplasm to the inner cytoplasm of the *feoB* mutant and wild-type cells, subcellular fractionation was performed by osmotic lysis using the periplastic lysing kit (Epicenter). Mid-log-phase grown cells were pelleted and washed in 10 mM Tris buffer (pH 7.4). Then the cells were resuspended in the uptake buffer to an OD_{600}=0.6 (equivalent to 10^9 bacteria/ml) and kept on ice. The uptake buffer was composed of 5 g/L Na_2HPO_4, 5 g/L KH_2PO_4, 1.18 g/L NH_4Cl, 0.089 g/L Na_2SO_4, 0.042 g/L $MgCl_2.6H_2O$ and 10 g/L casamino acids. The resuspended cells were allowed to equilibrate at 37˚C for 10 minutes before initiating the assay by $^{55}Fe^{2+}$ iron source (prepared as described earlier) was added to a final

concentration of 0.036 μM and samples (1 ml) were taken every 2 minutes. The cells were pelleted by centrifugation at 13000 rpm for 2 minutes. Then the cells were washed twice using 0.1 M citric acid and lysed by resuspending in 50 μl of periplastic lysing buffer and incubating at room temperature for 5 minutes. Then 50 μl of cold water was added to the lysed cells, mixed by inversion and incubated on ice for 5 minutes. The lysed cells were centrifuged for 5 minutes and the aqueous layer (periplastic fraction) was aspirated. The pellet was washed twice using 0.1 M citric acid and resuspended in 500 μl of cold water constituting the spheroplastic (cytoplasmic) fraction. Both the fractions were placed in 5 ml scintillation cocktail and counted as described previously.

$^{55}Fe^{2+}$ accumulation and growth assays

The accumulation of $^{55}Fe^{2+}$ iron and the growth kinetics were compared between the *feoB* mutant and the wild-type strains by growing them separately in MEMα medium in the presence and absence of radiolabeled Fe(II) iron source, provided at a final concentration of 0.036 μM. The radiolabeled Fe (II) iron source was prepared as described earlier. Then, 250 μl of the diluted Fe (II) source was added to 25 ml of starting culture. Sodium pyruvate (20 mM) was added as a carbon source to all the cultures. The gowth kinetics of the *feoB* mutants and the wild-type strains were monitored at 0, 8, 12 and 24 hours after the addition of the ferrous iron source (0.036 μM) by measuring the OD_{600} per ml at the above time points. In addition to monitoring the accumulation of ferrous iron in whole cells, the actual ferrous iron accumulation in the periplamisc and spheroplastic fractions were determined as described previously, by collecting samples (1 ml) at 0, 8, 12 and 24

hours. The data was statistically analyzed for all the $^{55}Fe^{2+}$ uptake assays and growth assays using the Student *t* test at a 5% level of significance.

In vivo colonization studies

Chick colonization assays

Two types of chick colonization assays were conducted: Non-competitive and competitive colonization.

Non-Competitive Chick colonization

One day old specific pathogen free chicks were obtained from Tyson farms, Arkansas. On arrival, cloacal swabs of the chicks were taken to ensure that they were *Campylobacter* free. Housing of chicks was maintained at 25˚C, and was provided with a brooder which maintained a temperature in the range of 33-35˚C. Chicks were given *ad libitum* commercial chicken starter diet and water. For challenge, *C. jejuni* wild-type and *feoB* mutant strains were grown in an MH biphasic medium in a microaerophilic chamber. The bacteria were harvested at mid-log-phase and resuspended in PBS buffer. Each chick was inoculated with 0.25 ml of a bacterial suspension which contained approximately 10^4 to 10^5 viable bacteria. An uninoculated group of birds was the negative control. Five birds per group were inoculated separately with the wild-type or mutant. Four days post challenge, the colonization potential was determined as per standard procedures described previously (17). After euthanasia, the ceca were collected, and the contents were homogenized and checked for viable counts by plating on plates of

Campylobacter agar base (Oxoid CM935) along with the *Campylobacter* selective Karmali antimicrobial supplements (Oxoid SR167E). Plates were incubated at 37˚C for 72 hours and the bacterial recovery titer was determined and expressed as CFU per gram of ceca. Non parametric Mann-Whitney rank sum test was used for statistical analysis at a 5% level of significance.

Competitive chick colonization

Five chicks were used per group for the competitive colonization studies. Each chick was inoculated with 0.5 ml of bacterial suspension containing a 1:1 mixture of wild-type and *feoB* mutant strains. The mixed inoculum contained approximately 10^5 viable bacteria each, of the wild-type and of the mutant, and was confirmed by plating serial dilutions of the mixed culture on MH agar with and without kanamycin (30 μg/ml). At 4 days post inoculation, the ceca were collected as described above. The cecal contents were homogenized, serially diluted in PBS buffer and were then plated onto *Campylobacter* agar base (Oxoid CM935), enhanced with the *Campylobacter* selective Karmali supplement (Oxoid SR167E) and on Karmali agar plate containing kanamycin (30 μg/ml). The plates were then incubated at 37˚C for 72 hours in a microaerophilic chamber before the colonies were counted. The mutant titer was obtained from the CFU recovered on Karmali agar plates containing kanamycin, and the wild type bacterial titer was calculated by subtracting the number of mutants from the total number of bacteria recovered on Karmali agar plates without kanamycin. Finally, the *in vivo* competitive index was calculated for each bird, which is the ratio of output mutant to wild-type bacteria recovered divided by the ratio of input mutant to wild type bacteria inoculated. A

single sample Student *t* test was used to statistically analyze the data at a 5 % level of significance.

Rabbit Ileal Loop (RIL) competitive colonization

The *in vivo* competitive index of the *feoB* mutant and the wild-type NCTC 11168 in the RIL model was determined as previously described (26). Rectal swabs were taken from the rabbits upon arrival to verify that they were *Campylobacter* free. Ileal loops were prepared according to the published protocols (5, 26). Briefly, two New Zealand White rabbits (<2 Kg, female) were anaesthetized, a laprotomy was performed, and two 20 cm sections of ileum with intact mesentric blood supply were ligated per animal. The four ileal loops (from 2 rabbits) were injected (each) with a 1 ml PBS suspension of a 1:1 mixed culture containing approximately 10^6 CFU/ml, each, of *C. jejuni* NCTC 11168 wild-type and *feoB* mutant strains. The 1:1 mixed inocula of the mutant and wild-type strains were confirmed by plating serial dilutions of this mixed culture on MH agar with and without kanamycin (30 μg/ml). The loops of 2 additional rabbits were inoculated with sterile PBS buffer and served as uninfected controls. After inoculating the intestinal loops, they were replaced in their appropriate position in the abdominal cavity, the abdominal wall and skin were closed as per standard procedures, and the rabbits were allowed to recover from anaesthesia. Forty-eight hours post inoculation, the rabbits were anaesthetized again, the intestinal loops were excised intact, and the animals were then euthanised. The contents of the loop as well as the mucous layer were collected and homogenized in 10 ml of PBS buffer. Serial dilutions of bacteria recovered from each rabbits' loop were plated on Karmali agar plates [*Campylobacter* agar base (Oxoid

CM935)] supplemented with the *Campylobacter* selective Karmali supplements (Oxoid SR167E) and Karmali agar plates containing kanamycin (30 μg/ml). Plates were incubated at 37˚C for 72 hours in a microaerophilic chamber before the colonies were counted. The titer of the mutant was obtained from CFU recovered on Karmali agar plates containing kanamycin, and the titer of the wild type was calculated by subtracting the number of mutants to the total number of bacteria recovered on Karmali agar plates without antibiotic. Finally, the *in vivo* competition index for each loop was calculated as described above for the competitive chick colonization studies. A Student *t* test was used to statistically analyze the data from the *in vivo* competition assays.

Piglet Colonization studies

Colostrum-deprived new born piglets were obtained from the Oklahoma State University swine farm, Stillwater,Oklahoma. The piglets, once farrowed, were cleaned with sterile towels and Betadine, and transported to the Laboratory Animal Research facility, OSU, Stillwater,Oklahoma. The experimental and uninoculated control piglets were kept in separate pens. The piglets were checked upon arrival with rectal swabs to verify that they were *Campylobacter* free. Each piglet was fed about 80-100 ml protein-rich, free of antibody and antibiotics reconstituted commercial replacer milk ration (Sav-A-Calf, Wisconsin) four times daily.

They were starved for about 2 hours prior to challenge with the mixed inocula. *C. jejuni* wild-type NCTC 11168 and *feoB* mutant strains were grown in biphasic MH medium to the mid-log-phase, centrifuged and resuspended in Similac commercial milk to an OD_{600} of approximately 2.0 (corresponding to $\approx 5 \times 10^{10}$ CFU/ml). Five ml (each) of the mutant

and wild-type strains were mixed at a 1:1 ratio. Two or three piglets (each) were fed the above mixed inocula orally. The mixed inocula were confirmed by plating serial dilutions on MH agar with and without kanamycin (30 μg/ml). Control piglets were fed milk without any bacteria.

All the piglets were observed daily for clinical signs such as fecal consistency, diarrhea and general health. The presence of blood in the feces was detected using EZ detection kit (Biomerica, USA). At 72 hours post inoculation, the piglets were first anesthetized with 0.25 ml/kg of ketamine (100 mg/ml) and 0.125 ml/kg of xylazine (20 mg/ml) (intramuscular), following which, they were euthanised with 0.25 ml/kg of Beuthenesia-D (intracardiac). Intestinal segments duodenum, jejunum, ileum, cecum and colon were recovered following necropsy. Gross lesions were examined in the piglets inoculated with the wild type strain alone. The contents and the mucous layer were collected from the intestinal segments and homogenized in 5 ml of PBS buffer. Serial dilutions of bacteria recovered from each organ were plated on *Campylobacter* selective media with and without antibiotics as described previously in the chick competitive colonization assay. The plates were then incubated at 37˚C for 72 hours in a microaerophilic chamber before the colonies were counted. The mutants and wild-type titer was determined and the competitive index was computed and statistically analyzed as described earlier in the chick competitive colonization assay.

In vitro Competition assay

A 1:1 mixture of mid-log-phase wild-type and mutant strains of *C. jejuni* from overnight cultures was taken in fresh MH medium and was used for the inoculation of three replicates of biphasic MH cultures. The measurement of OD_{600} over time was used to monitor the bacterial growth. Determination of the titer of each strain by plating on MH agar and MH agar containing kanamycin (30 μg/ml) was done in the early stationary phase and in the inoculum. The *in vitro* competitive index, which is the ratio of mutant to wild-type strain, was calculated for three independent growth experiments. The data was analyzed using the Student *t* test.

Ferric Reduction Assay

The ability of *C. jejuni* NCTC 11168 wild-type strain and *feoB* mutant to reduce a ferric iron source was assessed using bathophenantrolin-disulfonate (BPDS, Sigma) as a chromogenic ferrous iron chelator as described by others (35). *C. jejuni* NCTC 11168 and *feoB* mutant strains were cultured overnight in 50 ml of iron chelated MH + DFO (20 μM) medium. Uninoculated medium which was incubated under similar conditions served as a reference. BPDS was added to the overnight inoculated and uninoculated media, to achieve a final concentration of 1 mM. $FeCl_3$, which served as a ferric iron source, was added, so as to reach a final concentration of 50 μM, following which, the media and cells were incubated at 37˚C for 1 hour. Aliquots of 1 ml were taken, and centrifugation was done to remove the cells. Subsequently, at 535 nm, the optical densities of the Fe(II)-BPDS complexes in the supernatant was measured. Quantification

of the Fe(III)-reduction activity was expressed as nanomoles of Fe(II)-BPDS complexes formed per hour per 10^9 cells. A molar extinction coefficient of 22,140 at 535 nm was used for the determination of the amount of Fe(II)-BPDS complex formed in the assay. The experiment was performed in triplicates.

Results and Discussion

Construction of *C. jejuni feoB* mutants

In order to determine the role of the *feoB* gene in iron acquisition and colonization of the host gastrointestinal tract, we mutated the *feoB* gene in *C. jejuni* NCTC 11168 by allelic exchange strategy. Since *C. jejuni* NCTC 11168 is poorly invasive (21), we also mutated the *feoB* gene in two other highly invasive clinical isolates, *C.jejuni* ATCC 43931 (TGH 9011) and *C.jejuni* 81-176 (20, 21). The correct construction of the mutants was confirmed by PCR (Figure 1).

The *feoB* mutant had no growth defect *in vitro*

In order to determine whether the *C. jejuni feoB* mutant was affected in its ability to grow *in vitro*, we compared the growth rate of the *feoB* mutant and the wild-type *C. jejuni* NCTC 11168 strains grown in MH biphasic medium (Figure 2) and iron limited MEMα medium (Figure 3). The MH biphasic growth kinetics experiments represent the phenotype of the *C. jejuni* strains in iron containing medium, whereas the growth experiments in MEMα medium represent the growth pattern in iron limited conditions. There was no significant difference observed between the *feoB* mutant and the wild-type *C. jejuni* NCTC 11168 strains at a 5% level of significance in any of the growth experiments. Similar growth patterns were observed with *C. jejuni* 81-176 and *C. jejuni* TGH 9011 wild-type and *feoB* mutant strains (data not shown). These data

suggest that the *feoB* mutant had no growth defect *in vitro*. Similar findings were observed in another gram-negative pathogen *Helicobacter pylori* (30). There was no significant difference between the wild-type and the *feoB* mutant strains of *H. pylori* when grown in brain heart infusion (BHI) supplemented with 5% fetal bovine serum (FBS). Also, the gowth rates of the wild-type and *feoB* mutant strains were similar when grown in iron depleted BHI FBS. It has been shown that the *feoB* mutant of *Shigella flexneri* grew less well than the wild-type, indicative of a slight *in vitro* growth defect (24) , whereas, the *feoB* mutant of *Salmonella typhimurium* was unaffected in the *in vitro* growth as compared to the wild-type strain (28). It has been documented in *E. coli* that the *feoB* mutant was not affected in growth rate compared to the wild-type strain (27).

$^{55}Fe^{2+}$ uptake assays

Transport of ferrous iron in *C. jejuni* is proton dependent

It has been demonstrated in *E. coli* and *H. pylori* that the active transport of iron against its concentration gradient requires energy (8). The protonophore CCCP has been shown to dissipate the proton motive force across the inner membrane and abolish the iron transport in *H. pylori* (30). Recently, it has been demonstrated that the membrane protein FeoB contains an intramolecular G protein, which is essential for Fe (II) uptake in bacteria (15). It has not been documented in *C. jejuni* whether the ferrous iron transport across the inner membrane is proton dependent, and in order to determine the proton dependency for ferrous iron uptake, we used the proton ionophore cyanide-m-chlorphenyl hydrazone (CCCP). Radiolabeled Fe (II) uptake (0.036 μM) was monitored after treatment of cells with 33 μM CCCP. As shown in Figure 4, the CCCP treated *C.*

jejuni cells were completely inhibited in iron transport, whereas the untreated cells were able to transport Fe(II) iron. This result suggests that the transport of the non-FeoB mediated ferrous iron in *C. jejuni* is proton dependent.

FeoB was involved in ferrous iron transport

The *C. jejuni* FeoB system has not been extensively studied. Till date, there is only a single report on *C. jejuni* FeoB characterization and its role in ferrous iron transport by Raphael *et al* (22). They reported that in contrast to other bacterial FeoB systems, *C. jejuni* FeoB does not play a role in ferrous iron transport as analyzed by $^{55}Fe^{2+}$ uptake assays. They found no significant difference in the wild-type strain and *feoB* mutant in iron transport with non-sequenced *C. jejuni* isolates, *C. jejuni* M129 and *C. jejuni* F38011. Although they used iron depleted uptake buffer for the ferrous iron uptake experiments, the cells used in their assay were not iron starved, as they were grown in MH, which is an iron containing medium. They also reported that the *feoB* and the wild type *C. jejuni* F38011 strains were identical with respect to invasion of INT-407 intestinal epithelial cells and survival within J774A.1 macrophages, and therefore concluded that FeoB was non-functional in *C. jejuni*.

In order to determine whether the FeoB phenotype differs with respect to the *C. jejuni* isolates used in the study and the culture conditions in which the cells were grown prior to performing the uptake assays, we re-investigated the role of FeoB in ferrous iron transport in the sequenced strain, *C. jejuni* NCTC 11168.

We assessed the ferrous iron uptake of the wild-type and *feoB* mutant strains with cells grown in MH medium and in MH containing the iron chelator desferriferrioxamine

(20 µM) to the mid-log-phase. The objective of using cells grown in iron limited and iron rich media was to test whether the iron in the growth medium influenced the kinetics of ferrous iron uptake. The uptake assay was performed as described in the materials and methods section. As seen in Figure 4, there was no significant difference in the uptake of ferrous iron between the wild-type and mutant strains grown in MH medium. These findings are similar to the findings reported by Raphael *et al* (22). It has been shown that the expression of iron acquisition systems in cells grown under iron limited conditions was notably up-regulated compared to cells grown in iron rich conditions (10, 17). Therefore, monitoring the ferrous iron uptake rates of the *feoB* mutant and the wild type strains grown in the presence and absence of iron would ensure proper expression of iron acquisition systems. We determined the ferrous iron transport with cells grown in the presence and absence of iron prior to initiating the ferrous iron uptake assays. Interestingly, the ferrous iron uptake performed with cells grown in iron restricted desferriferrioxamine treated MH medium exhibited a significant difference ($p<0.009$) between the *feoB* mutant and the wild-type strains (Figure 5), with a decreased ferrous iron uptake in the *feoB* mutant.

Subcellular fractionation reveals cytoplasmic membrane transport

The uptake of ferrous iron requires only a cytoplasmic membrane transporter (29). Neither the specialized outer membrane receptors, nor energy is required for the entry of ferrous iron into the periplasm (31).

In order to determine whether the transport of Fe (II) iron from the periplasm to the cytoplasmic space is FeoB mediated in *C. jejuni*, we assessed the cellular fractions

(periplasmic and spheroplastic), for the accumulation of ^{55}Fe following osmotic lysis of the cells. The fractionation of whole cells into the periplasmic and spheroplastic fractions was confirmed by scanning electron micrograph (data not shown). *C. jejuni* wild-type strain and the *feoB* mutant were grown overnight in desferriferrioxamine (20 µM) treated MH medium to mid-log-phase and then assessed for iron transport. As shown in Figure 5, the *feoB* mutant had significantly lesser cytoplasmic $^{55}Fe(II)$ levels than the wild-type, indicating the *feoB* mutant to be defective in ferrous iron transport. On the other hand, the wild-type strain was demonstrated to have functional FeoB system since there was significantly higher $^{55}Fe(II)$ levels in the spheroplast (cytoplasmic fraction), suggestive of ferrous iron uptake from the periplasm to the inner cytoplasmic membrane. This subcellular fractionation data (Figure 5) is similar to the data from non-fractionated ferrous iron uptake experiments and therefore corroborates FeoB to be functional in the wild-type strain. However, these findings (Figure 5) are not in agreement with the ferrous iron transport studies reported by Raphael *et al*. This difference is therefore likely due to different growth conditions and different *C. jejuni* isolates used in the two studies.

The *feoB* mutant accumulates less iron when grown under iron-replete conditions

In order to analyze if the reduced iron uptake rates affected the iron accumulation in the *feoB* mutant, we determined the accumulation of $^{55}Fe^{2+}$ iron of the wild-type NCTC 11168 and the *feoB* mutant strains by performing $^{55}Fe^{2+}$ accumulation assays as described in the materials and methods section. The wild-type and *feoB* mutant strains were grown separately in MEMα medium with $^{55}Fe^{2+}$ iron at a final concentration of 0.036 µM. One

ml aliquots were collected at regular intervals and the iron accumulation was monitored as a function of the optical density at 600 nm (Figure 6A). In addition, uptake was determined following cell fractionation (Figure 6 B), similar to the previous experiment. As shown in Figure 6, the wild-type strain contains more radiolabeled iron than the *feoB* mutant at the mid-log and at early stationary phases. Statistical analysis by Student *t* test of the mid-log-phase samples shows that there is a trend for the NCTC and the *feoB* mutant strain to be different with respect to iron accumulation over time (p=0.08). At early stationary phase, there is a significant difference at a 5 % level of significance. The fractionated counts at mid-log-phase further corroborated the fact that the accumulation of $^{55}Fe^{2+}$ iron in the spheroplastic fraction of the wild type was higher than that of the *feoB* mutant, indicative of FeoB mediated iron uptake. In the *feoB* mutant, there was 81 % lower $^{55}Fe^{2+}$ iron accumulation in the spheroplastic fraction of the mutant compared to the wild type (p=0.03). Therefore, this significant decrease in iron accumulation in the *feoB* mutant cells can be attributed to the disruption of the FeoB inner membrane protein. Velayudhan *et al*. have shown similar results with the *feoB* mutant in *H. pylori*. By atomic absorption spectrophotometry, they demonstrated approximately 4-fold lower cellular iron levels in the mutant compared with the wild-type strain.

Role of FeoB in *C. jejuni* intracellular survival within eukaryotic cells

Although the importance of iron as an essential nutrient for *C. jejuni* metabolism has been previously shown (10, 17, 26, 29), however, the role of iron in intracellular survival of *C. jejuni* within eukaryotic cells is not known. Studies related to intracellular survival of *C. jejuni* within eukaryotic cells have been done to assess the role of catalase and

superoxide dismutase (4, 19). The intracellular iron requirements of *C. jejuni* within the host and its available sources are not exactly known. It has been conjectured that following internalization, *C. jejuni* should be able to survive and replicate intracellularly (4, 19). In order to determine if FeoB plays a role in intracellular iron acquisition for *C. jejuni*, we compared the survival of the *feoB* mutant and the wild-type *C. jejuni* 81-176 within cultured epithelial cells.

Intracellular survival assay was performed as described in materials and methods section. The INT-407 and IPEC-1 cells were infected with *C. jejuni* 81-176 and the *feoB* mutant at a MOI of 10:1 (number of bacteria per eukaryotic cell) and survival kinetics were analyzed over a 72 hour period (Figure 7A, 7B). The wild-type strain and the *feoB* mutant showed a decline in intracellular survival until 48 hours. There was no significant difference in the survivability of the wild type as compared to the mutants ($p>0.4$). Interestingly, between 48 hours to 72 hour period, the wild-type strain exhibited an increase in the recovery of intracellular bacteria, indicating its ability to persist and even multiply within INT-407 and IPEC-1 cells, whereas the number of *feoB* mutant continued to decline. The *feoB* mutant was approximately 5 fold and 8 fold more sensitive than the wild type strain in its ability to survive within IPEC-1 and INT-407 cells ($p<0.0001$) respectively, at a 5% level of significance. These results suggest that FeoB likely contributes towards *C. jejuni* intraepithelial survival and replication. It is likely that within 24-48 hours post infection, the majority of the cellular nutrient reserves may have been depleted, thus producing an acute shortage of iron for the bacterial metabolism. These findings suggest that under extremely limited iron conditions, only high affinity

iron transporters such as FeoB enable the iron starved bacteria to persist and multiply within the infected eukaryotic cells.

In addition to the intracellular survival assays, we also tested the binding and invasion efficiencies of the *feoB* mutant and the wild-type strains as a control to determine if FeoB played any role in the binding and invasion of *C. jejuni* to the host epithelial cells.

FeoB is not involved in binding and invasion of epithelial cells

It has been demonstrated that adhesion and invasion of the host mucosal surface are essential steps in the pathogenesis of *Campylobacter* gastroenteritis (11, 14, 34). However, poorly invasive *C. jejuni* strains have also been reported to heavily colonize the chicken ceca and the rabbit ileal loop (17, 26). This highlights the pathogenesis of *C. jejuni* infection to be a complex process.

In order to determine if FeoB plays a role in adhesion and invasion, we assessed the binding and invasion efficiency of *feoB* mutants and the wild-type using INT-407 human epithelial cells and IPEC-1 porcine epithelial cells. INT-407 cells have been commonly reported in adhesion and invasion studies of *C. jejuni* (21). IPEC-1 is an epithelial cell line derived from neonatal piglets. This is the first report where IPEC-1 cells have been used to study the *C. jejuni* virulence and colonization determinants. The objective of using IPEC-1 cells along with INT-407 cells was to correlate the findings from these *in vitro* experiments with the *in vivo* characterization of *feoB* mutants in neonatal piglets.

Adhesion and invasion assays were performed as described in materials and methods section. INT-407 and IPEC-1 cells were infected with *C. jejuni* wild-type and *feoB* mutants, with a multiplicity of infection (MOI) of 10:1 (number of bacteria per eukaryotic cell). Table 3 summarizes the findings of adhesion and invasion assays with INT-407 cells by comparing the wild-type *C. jejuni* strain and the *feoB* mutants. As previously shown, *C. jejuni* NCTC 11168 was found to poorly adhere and invade the INT-407 cells, whereas, *C. jejuni* TGH 9011 and *C. jejuni* 81-176 had higher percent binding and percent invasion efficiencies (21). Figure 7 A and B also shows binding and invasion efficiencies of the *feoB* mutant and the wild type *C. jejuni* 81-176 strain along with the intracellular survival. The *feoB* mutants and the wild-type *C. jejuni* strains had no significant differences ($p>0.2$) in binding and invasion efficiencies at a 5% level of significance.

Table 4 summarizes the binding and invasion assays with IPEC-1 cells comparing the wild-type strains and the *feoB* mutants of *C. jejuni* TGH 9011 and *C. jejuni* 81-176. There was no significant difference in the binding and invasion efficiencies of the *feoB* mutant and wild-type ($p>0.34$) at a 5% level of significance. The binding and invasion efficiencies of *C. jejuni* TGH 9011 and *C. jejuni* 81-176 with IPEC-1 cells were comparable to the corresponding efficiencies with INT-407 cells. This indicates that binding and invasion of *C. jejuni* isolates are independent of the cell lines used in the *in vitro* studies.

Experimental design and analysis of *feoB* microarray.

It has been shown that the genes involved in iron metabolism are expressed under iron limited conditions (1, 10, 17). In order to investigate the global changes in the gene expression profile elicited in response to iron limitation of the *feoB* mutant and the *C. jejuni* NCTC 11168 wild-type strains, we studied their transcriptome profiles when grown to mid-log-phase under iron-limited conditions in MEMα medium. The MEMα has been previously used to grow *C. jejuni* for microarray studies associated with iron metabolism (17). In this experiment, total RNA was purified from mid-log-phase grown wild-type strain and *feoB* mutant under iron-limited conditions. The RNA was reverse transcribed and fluorescently labeled as previously described (17, 25). Finally, the relative abundance of gene transcripts was compared for the wild type strain and *feoB* mutant using a *C. jejuni* NCTC 11168 microarray. The experiment was repeated three times (three biological replicates) and each experiment had three observations. So there were nine observations per gene. The data was normalized, merged and reported as the $\log_2$ ratio of the transcript abundance of *C. jejuni feoB* mutant (Cy5) grown in iron-starved conditions to that of the wild type strain (Cy3).

The statistical significance of the differential abundance of transcripts was analyzed by a Student *t* test. Genes were considered to be differentially expressed if the 'P' value was less than 0.01 and a minimum of two-fold change in the transcript abundance was found. Overall, 110 genes were differentially expressed between the *feoB* mutant and the wild-type strain. Table 5 summarizes the trend of these differentially expressed genes. Only 10 genes were found to be 2 to 4 fold up-regulated ($p<0.06$) (Table 6). The gene

involved in oxidative stress defense *katA* was 2-fold up-regulated. Expression of Cj0012c, which encodes a non-heme iron protein was 4-fold up-regulated. Components of cytochrome b6 (*petA, petB*) and a putative periplasmic protein were also up-regulated in the *feoB* mutant. Surprisingly, our experiments did not identify many iron acquisition genes that were reported in earlier studies to be up-regulated in iron-limited conditions. Also, our experiments revealed down-regulation of genes belonging to the flagellar biogenesis. These findings are in contrast to the findings reported in the microarray studies of *C. jejuni*'s response to iron limitation (17). This differential expression profile of the flagellar biogenesis genes in the *feoB* mutant with respect to iron starvation is unclear and requires further investigation. This highlights the complex nature of iron transport systems in *C. jejuni*.

The observed up-regulation of *katA* and other genes which are not involved in iron acquisition suggest that this could be a secondary regulatory response of *C. jejuni*, signaling iron restricted state of the *feoB* mutant. The *katA* gene and other oxidative stress defense genes such as *ahpC* and *PerR* have also been identified to be up-regulated under iron limited conditions *in vitro* (10, 17). Previous reports have shown that *C. jejuni katA* mutant was affected in its survival ability within the macrophages (4). Some of the hypothetical proteins and putative periplasmic proteins were up-regulated in the *feoB* mutant strain. It might be possible that the transcription of these genes was altered due to physiological and metabolic changes that take place in iron starved conditions.

Extensive screening of *C. jejuni feoB* mutants for colonization and pathogenicity testing in three animal models

Although the development of several animal models mimicking the *C. jejuni* pathogenesis have been cited in the literature, reports of their utilization in assessing the disease or colonization potential factors are very limited (16). Here we report the colonization potential of *C. jejuni feoB* mutants from three isolates based on competitive and non-competitive gut colonization testing in three diverse animal models; chicks, colostrum-deprived neonatal piglets and rabbits.

The chick model is a well characterized colonization model and has been used to screen the colonization potentials of *C. jejuni* mutant strains (7, 17). *C. jejuni* colonizes the intestine of chicks as commensals (9). As there are no clinical signs observed in chicks challenged with *C. jejuni,* the chicks serve solely as a colonization model.

The digestive tract of neonatal piglets is similar to the human gastrointestinal tract (2, 16). Use of colostrum-deprived new born piglets as an *in vivo* model for human Campylobacterioisis has been documented by Babakhani *et al* (2). They observed diarrhea in experimentally infected piglets characterized by the presence of mucous and blood, as seen typically in human Campylobacterioisis. Therefore, we chose to use this model to screen the *C. jejuni feoB* mutant by competitive colonization assay. However, till date, there are no reports documenting the use of colostrum-deprived neonatal piglets in the screening of *C. jejuni* mutants.

The RIL model has also been shown to be a suitable model for human Campylobacteriosis as characteristic histopathological lesions were observed in the gut of experimentally infected rabbits (5). Signs of intestinal distention due to increased accumulation of fluid and gas was previously reported in RIL studies (26). We have recently reported the rabbit ileal loop model for screening of *C. jejuni* mutants by competitive colonization (26).

The feoB mutant is significantly affected in colonization of chicken ceca

Colonization potential of the *feoB* mutants of *C. jejuni* NCTC 11168, *C. jejuni* TGH 9011 and *C. jejuni* 81-176 were assessed by non-competitive and competitive colonization in chicks.

Non-competitive cecal colonization

The colonization potential of *feoB* mutants and wild-type *C. jejuni* strains were determined by orally challenging 5 birds per group separately with approximately 10^4 to 10^5 viable bacteria. Five uninoculated birds served as negative control. Four days post inoculation, chicks were euthanized and ceca were recovered. Bacterial recovery was calculated for every bird per gram of ceca. The minimal limit of detection for this assay was 100 cfu/g ceca. As shown in Figure 8, all the three *feoB* mutants were significantly attenuated in their ability to colonize the chick ceca. These results indicate the importance of *feoB* in cecal colonization by *C. jejuni*. This *in vivo* phenotype of *C. jejuni feoB* mutant is similar to the *feo* mutant phenotype of enteric pathogens *E. coli* and

Salmonella enterica which were reported to be defective in the intestinal colonization of mice (27, 28).

The competitive chick colonization assays

The *feoB* mutants of *C. jejuni* NCTC 11168 and *C. jejuni* TGH 9011 were also tested for their colonization potential *in vivo* by competitive colonization in chicks. Each *feoB* mutant and the corresponding wild-type strain was independently mixed in a 1:1 ratio and orally challenged to 5 birds. Mixed inocula contained approximately 10^5 viable bacteria of each type and the inoculum titer was confirmed by plating the serially diluted inoculum on MH plates with and without kanamycin (30μg/ml). Four days post inoculation, the birds were euthanized and the cecal contents were collected, diluted serially and plated on *Campylobacter* selective medium with and without kanamycin (30μg/ml) to enumerate the number of *feoB* mutant and wild-type strain. Then, the competitive index (C.I.) was calculated as the ratio of the output mutant to the wild-type strain recovered divided by the ratio of input mutant to the wild-type bacteria inoculated per bird. Interestingly, as shown in figure 9, both the *feoB* mutants were significantly affected in their colonization abilities ($p<0.0001$), based on a single sample Student *t* test at a 5 % level of significance. The outcome of *in vivo* chicken competitive colonization assays was similar to the outcome of *in vivo* chicken non-competitive assays. Therefore, *feoB* mutant was significantly affected in its colonization potential.

The *C. jejuni* infected colostrum-deprived neonatal piglets produce symptoms associated with the disease

We successfully established the neonatal piglet model by demonstrating substantial colonization levels with each of the three *C. jejuni* isolates, *C. jejuni* NCTC 11168, TGH 9011 and 81-176. Approximately 10^4 cfu per gram of intestine were recovered when these piglets were orally challenged with approximately 10^{10} cfu. All the isolates equally colonized the intestine of piglets (data not shown). The challenged piglets developed diarrhea within 24 to 48 hours. The feces were semi-solid to watery in consistency, and were characterized by the presence of blood and mucus in the piglets challenged with *C. jejuni* TGH 9011 and *C. jejuni* 81-176 isolates. No blood was observed in the feces of piglets challenged with *C. jejuni* NCTC 11168 isolate. The non-infected control piglets did not have any diarrhea. These findings were consistent with the findings of Babakhani *et al* (2). Histopathological analyses revealed no or very mild gross lesions consisting of hyperemia and patches of petechial hemorrhages. Therefore, in contrast to the study from Babakhani *et al*, no severe pathological lesions were observed. This difference seen in pathogenesis could be a result of host specificity, inoculum or differences in the *C. jejuni* isolates used in the two studies.

The *feoB* mutant of all the *C. jejuni* isolates tested were significantly attenuated in competitive colonization of intestine of piglets

The *feoB* mutants from all the three strains, *C. jejuni* NCTC 11168, *C. jejuni* TGH 9011 and *C. jejuni* 81-176 were tested with their corresponding wild-type strain in 3, 3 and 2 piglets respectively for *in vivo* competitive colonization. Housing and management of the

colostrums-deprived neonatal piglets used in this study has been described in the materials and methods section.

C. jejuni wild-type and *feoB* mutant strains were grown in biphasic MH medium to mid-log-phase, centrifuged and resuspended in 5 ml Similac milk (commercial formula) each for every piglet. Approximately 5 X 10^{10} cfu viable bacteria per ml was the strength of each inoculum. Both the inocula were mixed and 10 ml of this mixed inocula was fed orally to each piglet. The mixed inocula was confirmed by plating serial dilutions on MH agar plates with and without kanamycin. Seventy-two hours post challenge, the duodenum, jejunum, ileum, cecum and colon portions of the intestinal segments were recovered. The gut contents along with the mucus were collected, resuspended in PBS buffer and serial dilutions of recovered bacterial suspensions were plated for enumeration on selective media for each organ as described in the chick competitive colonization assay. The mutant and the wild-type titer were determined and the competitive index (C.I) calculated for each organ. One-sample Student *t* test statistical analysis was performed at a 5% level of significance. The competitive indices for all the intestinal segments were calculated as the ratio of the output mutant and wild-type strain recovered divided by the ratio of the input mutant and wild-type strain inoculated. For all the organs, the p value was <0.0001, indicating that the *feoB* mutant of *C. jejuni* NCTC 11168 strain was significantly affected in colonizing the gastrointestinal tract of piglets (Figure 10A). Similarly, the colonization potentials of the *feoB* mutants of *C. jejuni* TGH 9011 (Figure 10B) and *C. jejuni* 81-176 (Figure 10C) strains were also significantly affected.

The *feoB* mutant of *C. jejuni* TGH 9011 was recovered from only one organ (cecum) of one piglet of the three piglets assessed for competitive colonization in the gut. In case of all of the other organs from the three piglets, the recovery of mutant bacteria was below the detection limit of our assay (100 cfu/gm) (Figure 10B). From these data, it is clear that *C. jejuni* FeoB plays a significant role in the colonization of the gastrointestinal tract of piglets.

The feoB mutant was compromised in competitive colonization of the in vivo rabbit ileal loop (RIL)

We determined the competitive colonization ability of *feoB* mutant and wild-type *C. jejuni* NCTC 11168 strain in the RIL model. Surgical procedures have been explained in materials and methods section. Four loops were inoculated with a mixed inocula containing approximately 1:1 ratio of wild-type strain and the mutant. Mixed inocula contained approximately 10^6 bacteria each of the wild-type and the *feoB* mutant strain and were confirmed by plating the serial dilutions on MH plates with and without kanamycin (30 μg/ml). Two ileal loops inoculated with sterile PBS buffer served as uninfected control. Upon 48 hours post infection, the loop contents were recovered and plated to enumerate the wild-type and mutant bacterial titer similar to the bacterial titer determination described in the *in vivo* competitive chick colonization assay.

The data was statistically analyzed using a Student *t* test and the *feoB* mutant was found to be significantly affected in its colonization ability ($p=0.0176$) at a 5% level of

significance (figure 11). The overall recovery of the *feoB* mutant in the RIL assay was more than the *feoB* mutant recovered in the chick and piglet *in vivo* competition assay. This difference in mutant colonization levels could be attributed to the increased dose of inoculum administered in the RIL model. Also, with the RIL model, the ileal loop is physically ligated and might favor the survival of mutants unable to efficiently colonize the gut as compared to the two other models, where there is no physical ligation and they are subjected to removal through peristalsis and mechanical flushing.

The *feoB* mutant is not affected in the *in vitro* competition growth assay

In order to determine whether the *in vivo* colonization defect of the *feoB* mutant was a specific characteristic feature of *in vivo* growth, we performed the *in vitro* competition growth assay. A 1:1 mixture of *feoB* mutant and the wild-type *C. jejuni* NCTC 11168 strain was mixed into fresh MH medium. The mixed inocula was plated on MH and MH containing kanamycin (30 μg/ml) to confirm the 1:1 ratio of the mutant and the wild-type strains. The cultures were grown under microaerophilic conditions at 37˚C until early stationary phase. The titer of the wild type and *feoB* mutant was then determined by plating on MH and MH containing kanamycin (30 μg/ml). The *in vitro* competition index (C.I) was determined by calculating the ratio of the mutant to the wild-type strain (Figure 11). The *feoB* mutant was found to be not significantly affected in its ability to grow in competition with the wild type strain ($p>0.648$) at 5 % levels of significance as determined by the Student *t* test. Consequently, the decreased ability of the *feoB* mutant to colonize the gut of all the animal models tested is an *in vivo* specific defect.

Cj1397 and Cj1398 (*feoB*) genes are co-transcribed

Cj1397 gene encodes a putative protein of 74 amino acids and bears 16% identity to the *E. coli* FeoA. Its role in iron transport is not yet known. Cj1398 (FeoB) appeared to be in an operonic structure with Cj1397 (13, 18). However, this genetic organization has not been experimentally demonstrated. By RT-PCR, we demonstrated that Cj1397 and Cj1398 are in an operonic structure. The RT-PCR reactions were performed using a combination of primers that annealed within the genes Cj1395, Cj1397 and Cj1398. Each reaction gave a product of the expected size, as shown in Figure 12. Cj1395, located upstream of Cj1397, was in the same orientation as Cj1397. Cj1395 was individually transcribed, however it was not co-transcribed with Cj1397. Cj1397 and Cj1398 genes were found to be co-transcribed. Cj1399c, located downstream of Cj1398, is in opposite orientation to that of Cj1398 and was therefore not checked. Therefore, these results confirmed that only Cj1397 and Cj1398 genes are co-transcribed.

C. jejuni possesses ferric iron reduction ability

Ferric reduction ability has been demonstrated in *H. pylori* (30, 35). In order to test whether the ferric iron reduction ability is present in *C. jejuni*, we performed ferric reduction assays as described in the materials and methods section. Using a specific chromogenic Fe(II) iron chelator, bathophenantrolin-disulfonate (BPDS) at 1 mM, we observed a ferric reduction activity of 16.68 ± 0.8 nM of BPDS-Fe^{2+} complex produced by 10^9 cells per minute by the wild type *C. jejuni* NCTC 11168 strain. There was no reduction of ferric iron observed in the cell-free controls. This data demonstrated that the

wild type strain *C. jejuni* NCTC 11168 possessed a ferric reduction ability, which suggests the presence of an unknown reductant or extracellular ferric reductase in *C. jejuni*. It is likely that the ferric iron source maybe reduced to ferrous iron before being transported through the inner cytoplasmic membrane in *C. jejuni*. A similar role of NAD(P)H:flavin oxido reductase in Fe(III) reduction was proposed by Coves *et al* (3). Worst *et al* have demonstrated in *H. pylori* that the *ribBA* locus, which encodes genes for riboflavin synthesis also causes ferric reduction and plays a role in iron acquisition (35).

The *feoB* mutant and the wild-type *C. jejuni* NCTC 11168 strain exhibit comparable ferric iron reduction activity

In order to compare the ferric iron reduction ability of the *C. jejuni* wild-type strain and the *feoB* mutant strain, we performed the ferric iron reduction assay as described earlier. A Fe^{3+} reduction activity of 16.68 ± 0.8 nmol and 16.27 ± 0.423 nmol of BPDS-Fe^{2+} complex were produced by 10^9 cells per minute by the wild-type strain and *feoB* mutant strain respectively. Therefore, the ferric reduction ability was not affected in the *feoB* mutant at a 5% level of significance as compared to the wild-type strain.

Concluding remarks

The role of FeoB in *C. jejuni* was assessed for iron acquisition and colonization of animal hosts. The *feoB* gene was knocked out by insertional mutagenesis in three *C. jejuni* isolates. *In vitro* characterization of these *feoB* mutants by intracellular survival assays (within porcine and human intestinal epithelial cells), FeoB microarray analysis, iron transport and accumulation experiments demonstrated that the FeoB protein is required for ferrous iron transport and that ferrous iron is an important intracellular iron source for *Campylobacter*. *In vitro* experimental findings were strongly supported by *in vivo* animal colonization experiments. This was the first study which documented the screening of *C. jejuni* mutants for their colonization potentials in the colostrum-deprived neonatal piglets along with the chicken ceca and rabbit ileal loop colonization. Consistently, the animal experiments revealed that the *feoB* mutant was attenuated in the *in vivo* colonization in all the animal models tested. Consequently, this study clearly indicates that FeoB mediated ferrous iron transport is essential for successful gut colonization by *C. jejuni*.

References

1. Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones. 2003. Bacterial iron homeostasis. FEMS Microbiol Rev 27:215-37.

2. Babakhani, F. K., G. A. Bradley, and L. A. Joens. 1993. Newborn piglet model for Campylobacteriosis. Infect Immun 61:3466-75.

3. Coves, J., B. Delon, I. Climent, B. M. Sjoberg, and M. Fontecave. 1995. Enzymic and chemical reduction of the iron center of the *Escherichia coli* ribonucleotide reductase protein R2. The role of the C-terminus. Eur J Biochem 233:357-63.

4. Day, W. A., Jr., J. L. Sajecki, T. M. Pitts, and L. A. Joens. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. Infect Immun 68:6337-45.

5. Everest, P. H., H. Goossens, P. Sibbons, D. R. Lloyd, S. Knutton, R. Leece, J. M. Ketley, and P. H. Williams. 1993. Pathological changes in the rabbit ileal loop model caused by *Campylobacter jejuni* from human colitis. J Med Microbiol 38:316-21.

6. Friedman, C. R., R. M. Hoekstra, M. Samuel, R. Marcus, J. Bender, B. Shiferaw, S. Reddy, S. D. Ahuja, D. L. Helfrick, F. Hardnett, M. Carter, B. Anderson, and R. V. Tauxe. 2004. Risk factors for sporadic *Campylobacter* infection in the

United States: A case-control study in FoodNet sites. Clin Infect Dis 38 Suppl 3:S285-96.

7. Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell. 2004. The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. J Bacteriol 186:503-17.

8. Hantke, K. 2003. Is the bacterial ferrous iron transporter FeoB a living fossil? Trends Microbiol 11:192-5.

9. Hendrixson, D. R., and V. J. DiRita. 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol 52:471-84.

10. Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells. 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology 151:243-57.

11. Hu, L., and D. J. Kopecko. 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. Infect Immun 67:4171-82.

12. Kammler, M., C. Schon, and K. Hantke. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J Bacteriol 175:6212-9.

13. Ketley, J. M. 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology 143 (Pt 1):5-21.

14. Kopecko, D. J., L. Hu, and K. J. Zaal. 2001. *Campylobacter jejuni*--microtubule-dependent invasion. Trends Microbiol 9:389-96.

15. Marlovits, T. C., W. Haase, C. Herrmann, S. G. Aller, and V. M. Unger. 2002. The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. Proc Natl Acad Sci U S A 99:16243-8.

16. Newell, D. G. 2001. Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models. Symp Ser Soc Appl Microbiol:57S-67S.

17. Palyada, K., D. Threadgill, and A. Stintzi. 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol 186:4714-29.

18. Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M.

Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-8.

19. Pesci, E. C., D. L. Cottle, and C. L. Pickett. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect Immun 62:2687-94.

20. Pickett, C. L., T. Auffenberg, E. C. Pesci, V. L. Sheen, and S. S. Jusuf. 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni.* Infect Immun 60:3872-7.

21. Poly, F., D. Threadgill, and A. Stintzi. 2004. Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. J Bacteriol 186:4781-95.

22. Raphael, B. H., and L. A. Joens. 2003. FeoB is not required for ferrous iron uptake in *Campylobacter jejuni*. Can J Microbiol 49:727-31.

23. Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol 54:881-941.

24. Reeves, S. A., A. G. Torres, and S. M. Payne. 2000. TonB is required for intracellular growth and virulence of *Shigella dysenteriae*. Infect Immun 68:6329-36.

25. Stintzi, A. 2003. Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. J Bacteriol 185:2009-16.

26. Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke. 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect Immun 73:1797-810.

27. Stojiljkovic, I., M. Cobeljic, and K. Hantke. 1993. *Escherichia coli* K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. FEMS Microbiol Lett 108:111-5.

28. Tsolis, R. M., A. J. Baumler, F. Heffron, and I. Stojiljkovic. 1996. Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. Infect Immun 64:4549-56.

29. van Vliet, A. H., J. M. Ketley, S. F. Park, and C. W. Penn. 2002. The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. FEMS Microbiol Rev 26:173-86.

30. Velayudhan, J., N. J. Hughes, A. A. McColm, J. Bagshaw, C. L. Clayton, S. C. Andrews, and D. J. Kelly. 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. Mol Microbiol 37:274-86.

31. Wandersman, C., and P. Delepelaire. 2004. Bacterial Iron Sources: From Siderophores to Hemophores. Annu Rev Microbiol.

32. Wassenaar, T. M., and M. J. Blaser. 1999. Pathophysiology of *Campylobacter jejuni* infections of humans. Microbes Infect 1:1023-33.

33. Wassenaar, T. M., and D. G. Newell. 2000. Genotyping of *Campylobacter* spp. Appl Environ Microbiol 66:1-9.

34. Wooldridge, K. G., and J. M. Ketley. 1997. *Campylobacter*-host cell interactions. Trends Microbiol 5:96-102.

35. Worst, D. J., M. M. Gerrits, C. M. Vandenbroucke-Grauls, and J. G. Kusters. 1998. *Helicobacter pylori ribBA*-mediated riboflavin production is involved in iron acquisition. J Bacteriol 180:1473-9.

36. Yao, R., R. A. Alm, T. J. Trust, and P. Guerry. 1993. Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. Gene 130:127-30.

Table 1: Bacterial strains and plasmids

Strain or Plasmid	Relevant characteristics[a]	Source or reference
E. coli DH5α	*endA1 hsdR17($r_K^- m_K^-$)supE44 thi-1*	Invitrogen
C. jejuni		
AS 144	*C. jejuni* NCTC 11168	NCTC
AS 53	*C. jejuni* ATCC 43431 (TGH 9011)	ATCC
AS 275	*C. jejuni* 81-176	Pickett
AS 217	AS 144 Δ *feoB::Km*^{r}	This study
AS 236	AS 53 Δ *feoB::Km*^{r}	This study
AS 237	AS 275 Δ*feoB::Km*^{r}	This study
Plasmids		
pILL 600	Km^r resistance gene	Labigne
pCAP	Cloning and suicidal vector, Amp^r	Roche
pAS 43	pCAP carrying *feoB*	This study
pAS 223	pCAP carrying Δ*feoB:: Km*^{r}	This study

[a] Km^r, kanamycin resistance gene; Amp^r , ampicillin resistant

Table 2: Primers used in this study

Primer	DNA sequence from 5' to 3' (restriction site) [a]
feo-1	CGCTGGCCAAAGTCATTGATTTGCCAGGAAC (*Mlu*NI)
feo-2	CGCTGGCCAGCCACTGCACTTGGTATAGG (*Mlu*NI)
Cj1395-F	CGATTGCGATCCTTTAGAAGCAAA
Cj1395-R	CGAGTGGTTTAAATTTTCTAGATTGATGA
Cj1397-*F*	CGACGCTCACAAAGAACTCAAAAA
Cj1397-R	CGAGCTTCATCAGATCTTAGGATAACACA
Cj1398-F	CGATCAGCCTAATGTAGGCAAAAGTC
Cj1398-*R*	CGATGCGTTCTAAATTGGTAGCATC

[a] The restriction sites used for cloning are underlined and correspondingly shown in parenthesis

Table 3: Binding and Invasion assays using the INT-407 cell line

	NCTC		TGH		81-176	
	Wild Type	Δ*feoB*	Wild Type	Δ*feoB*	Wild Type	Δ*feoB*
% Binding[a]	0.0043 ± 0.001	0.003 ± 0.0007	1.39 ± 0.18	1.49 ± 0.19	1.71 ± 0.11	1.49 ± 0.13
% Invasion[b]	0.00036 ± 0.000087	0.00035 ± 0.000087	0.37 ± 0.064	0.38 ± 0.053	0.67 ± 0.08	0.63 ± 0.06

[a] obtained by subtracting the number of invaded bacteria from the total number of bacteria recovered without gentamicin treatment

[b] percentage of inoculum recovered after gentamicin treatment

Table 4: Binding and Invasion assays using the IPEC-1 cell line

	NCTC		TGH		81-176	
	Wild Type	Δ*feoB*	Wild Type	Δ*feoB*	Wild Type	Δ*feoB*
% Binding[a]	ND[c]	ND[c]	1.43 ± 0.1	1.57 ± 0.13	1.43 ± 0.13	1.37 ± 0.12
% Invasion[b]	ND[c]	ND[c]	0.3 ± 0.033	0.28 ± 0.046	0.62 ± 0.08	0.49 ± 0.09

[a] obtained by subtracting the number of invaded bacteria from the total number of bacteria recovered wihtout gentamicin treatment

[b] percentage of inoculum recovered after gentamicin treatment

[c] Not Determined

Table 5: Summary of genes differentially expressed

Number of genes	Fold change	Genes of interest
a) Up-regulated in Δ*feoB*		
20	1.5-4.0	*ExbB2*, *hrcA*, *hspR*, *ceuE* and all the genes listed in Table 6.
23	1.2-1.5	*ExbB2*
b) Down-regulated in Δ*feoB*		
25	2.5 and above	Flagellar biosynthesis genes: *FlgD*, *FlgE*, *FlgE2*, *FlgL*, *FlgB*
15	1.2-2.0	*ExbB3*, Cj020, Cj0203

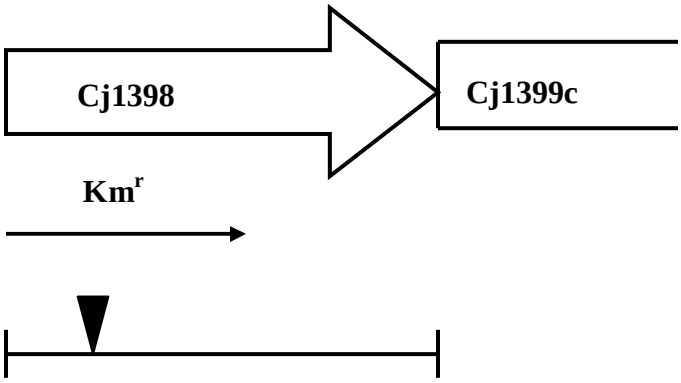
Table 6: *feoB* microarray analysis

Genes up-regulated in Δ*feoB*	Fold change	P value	Function
katA	2.0	0.00643	Oxidative stress defense
petA	2.6	0.00418	Cytochrome b6 component
petB	2.0	0.00114	Cytochrome b6 component
Cj1169c	2.0	9.25E-4	Putative periplasmic protein
Cj0012c	3.85	5.94E-4	Non-heme iron protein
Cj0262c	2.0	1.4E-5	Putative signal transduction protein
ppi	2.0	9.75E-4	Peptidyl-prolyl-cis-trans isomerase
rpsP	2.0	0.00376	30S ribosomal protein
aat	2.0	0.00542	Putative leucyl/phenylalanyl t-RNA protein transferase
Cj1710c	3.4	1.8E-4	Hypotheical unknown protein

Figure 1: Mutagenesis strategy of the *feoB* gene in *C.jejuni* NCTC 11168 and mutant verification. **A)** An *feoB* mutant was constructed by the insertion of a Km^r cassette into a unique ClaI site within the *feoB* gene. The distance from the translational start codon of the gene to the Km^r cassette insertion site is indicated. **B)** 0.9% agarose gel of PCR product obtained with the wild type *C.jejuni* NCTC 11168 (lane 2), the *feoB* mutant (lane 3) and standard 1Kb DNA ladder (lane 1).

Figure 1:

A)



734 bp
ClaI

B)

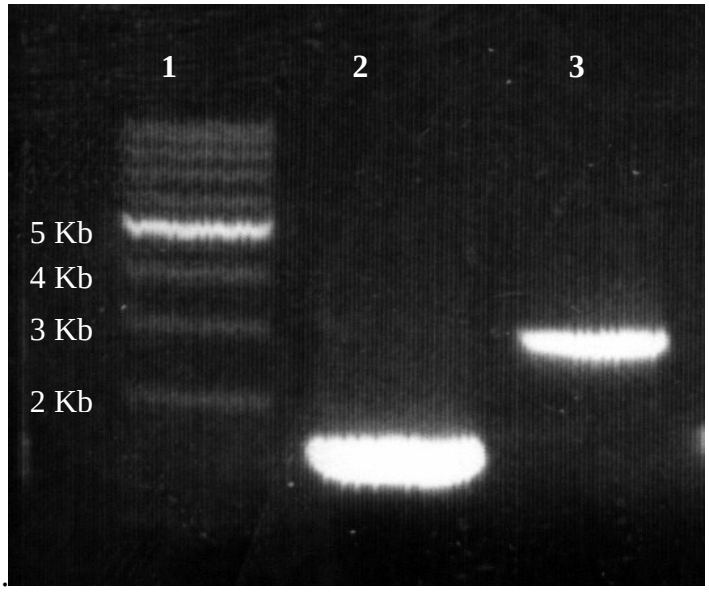


Figure 2: Comparitive growth of *C. jejuni* NCTC 11168 wild-type and *feoB* mutant in MH biphasic growth medium. *C. jejuni* starter cultures were grown overnight in MH biphasic medium and were then inoculated into fresh MH biphasic flasks. The data represents the mean of three independent experiments ± standard error.

Figure 2:

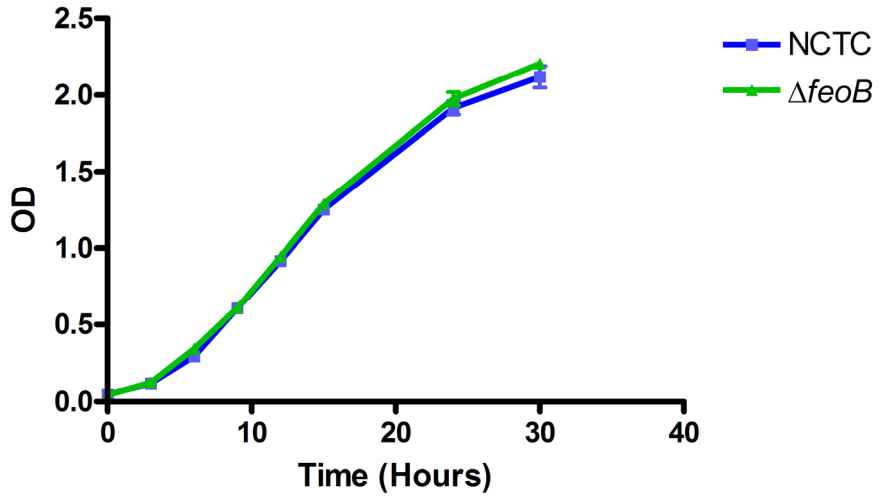


Figure 3: Growth of *C. jejuni* NCTC 11168 wild-type and *feoB* mutant in MEMα (supplemented with 20 mM sodium pyruvate as an additional carbon source) in the presence or absence of $^{55}Fe^{2+}$ [0.036 μM final concentration]. Strains were pre-cultured overnight in MH biphasic medium, harvested, washed and resuspended in MEMα before inoculating into fresh MEMα with and without radiolabeled iron. The data is from two independent experiments and represents the mean ± standard error. Since radiolabeled iron was reduced to ferrous iron using sodium ascorbate, sodium ascorbate was also added in equimolar concentration to MEMα culture, grown without an iron source.

Figure 3:

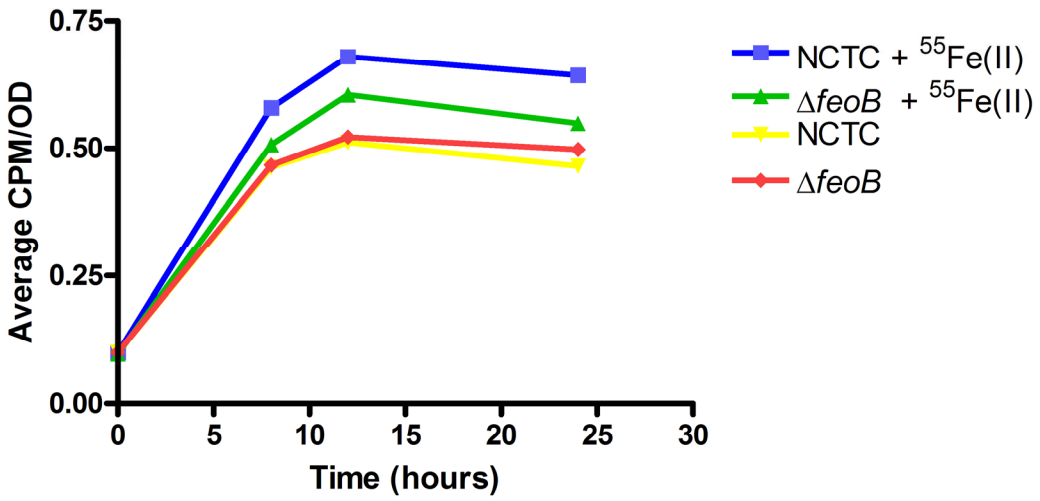

0.75
0.50
0.25
0.00
Average CPM/OD
0
5
10
15
20
25
30
Time (hours)
NCTC + 55Fe(II)
ΔfeoB + 55Fe(II)
NCTC
ΔfeoB

Figure 4: ^{55}Fe (II) uptake of *C. jejuni* NCTC 11168 and *feoB* mutant with cells grown in MH medium. 0.036 µM was the final concentration of radiolabeled Fe (II) used in the assays. Proton dependent and energy dependency for uptake was demonstrated by addition of 33 µM CCCP and by performing uptake at 0ºC. The experiment was repeated twice, and the data represents the mean ± standard error.

Figure 4:

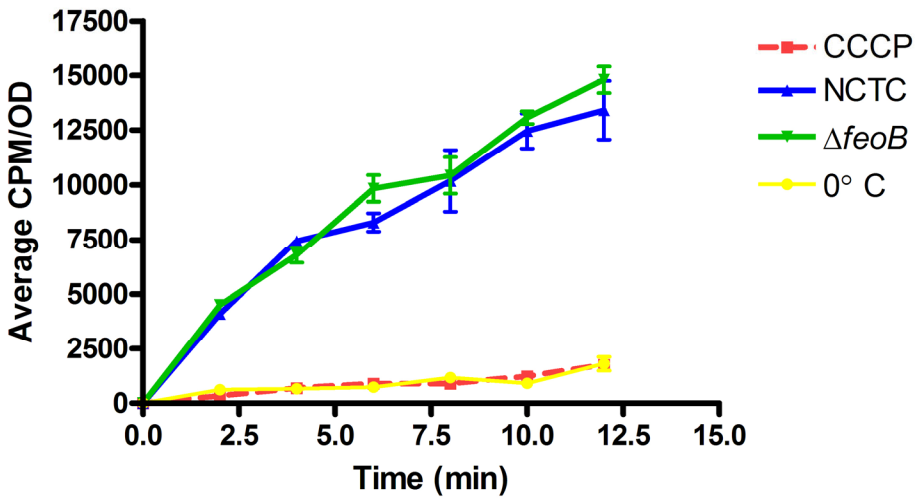
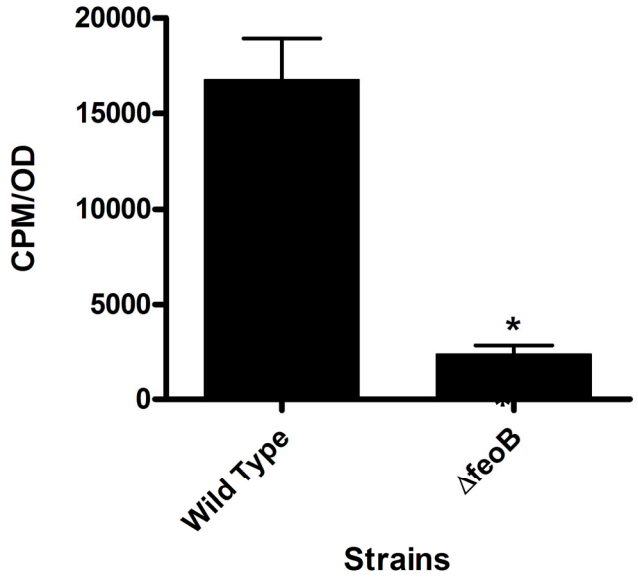

Average CPM/OD
17500
15000
12500
10000
7500
5000
2500
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time (min)
CCCP
NCTC
ΔfeoB
0° C

Figure 5: Whole cell $^{55}Fe(II)$ uptake assay for *C. jejuni* wild-type strain and *feoB* mutant. **A)** Cells were grown overnight to mid-log-phase in iron chelated medium (MH+DFO) [20 μM concentration] and uptake of radiolabeled ferrous iron was assessed 15 minutes after the addition of iron (0.036 μM) for the whole cells. The data represents the means of two independent experiments ± standard error. **B)** Cells were grown overnight upto mid-log-phase in iron chelated medium (MH+DFO) [20 μM concentration] and uptake of radiolabeled ferrous iron was assessed 15 minutes after the addition of iron (0.036 μM) for the sub cellular fractions obtained by osmotic lysis post uptake. The data represents the means of two independent experiments ± standard error. * indicates significant difference at a 5% level of significance

Figure 5

A)


20000
15000
10000
5000
0
CPM/OD
*
Wild Type
ΔfeoB
Strains

B)

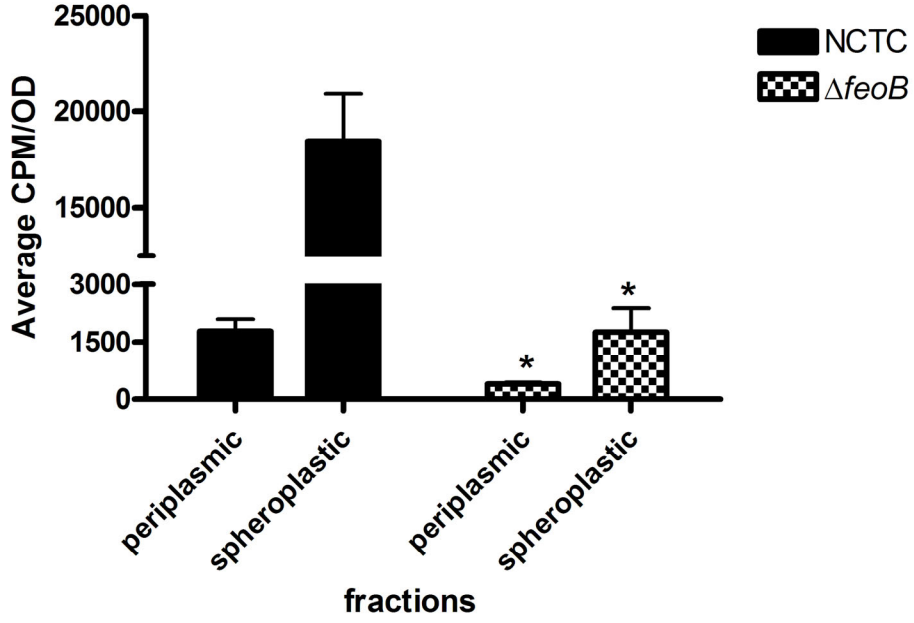
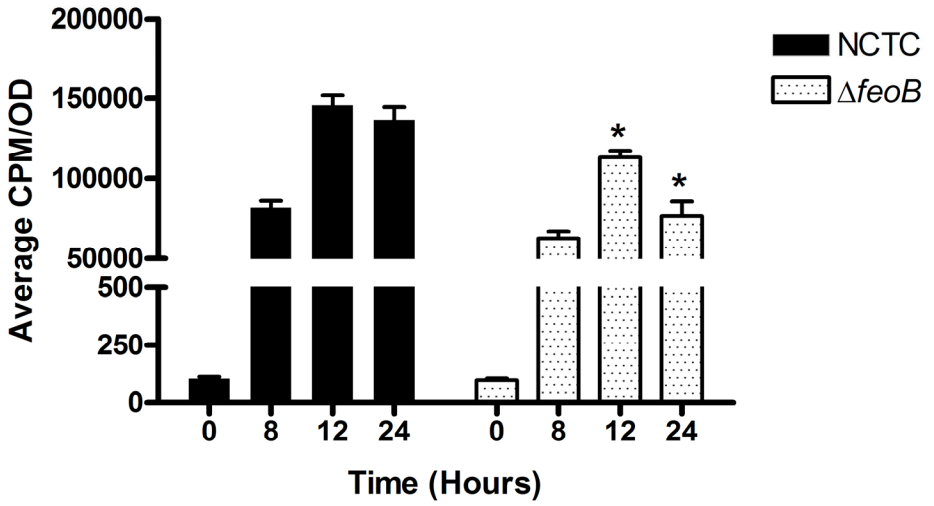

25000
20000
15000
3000
1500
0
Average CPM/OD
NCTC
ΔfeoB
*
*
periplasmic
spheroplastic
periplasmic
spheroplastic
fractions

Figure 6: Whole cell ^{55}Fe(II) accumulation in *C. jejuni* wild-type and *feoB* mutant grown in iron-replete conditions. **A)** The wild-type and *feoB* mutant strains were grown separately in MEMα containing 20 mM sodium pyruvate + 0.036 μM ^{55}Fe(II) final concentration, over a period of 24 hours. The accumulation of iron by the two strains was measured at 0, 8, 12 and 24 hours, similar to the ^{55}Fe(II) uptake assays described previously. The data represents the mean of two experiments ± standard error. **B)** The wild-type and *feoB* mutant strains were grown separately in MEMα containing 20 mM sodium pyruvate + 0.036 μM ^{55}Fe(II) final concentration, over a period of 24 hours. The accumulation of iron by the two strains was measured at mid-log-phase (12 hours) similar to the ^{55}Fe(II) uptake assays described previously, to determine the levels in spheroplastic and periplasmic fractions post uptake. The data represents the mean of two experiments ± standard error. * indicates significant difference at a 5% level of significance.

Figure 6

A)


200000
150000
100000
50000
500
250
0
Average CPM/OD
0
8
12
24
0
8
12
24
Time (Hours)
NCTC
ΔfeoB
*
*

B)

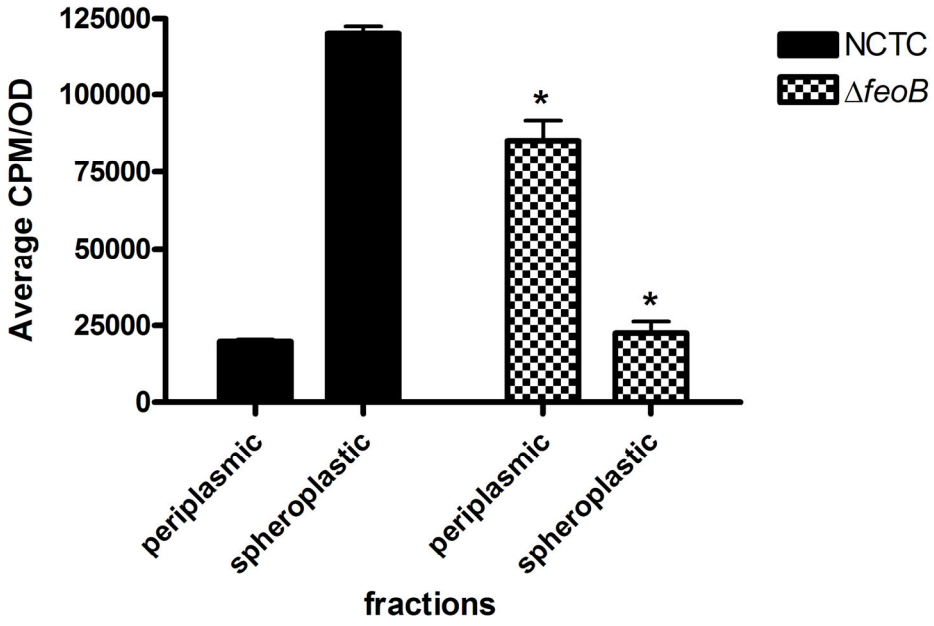
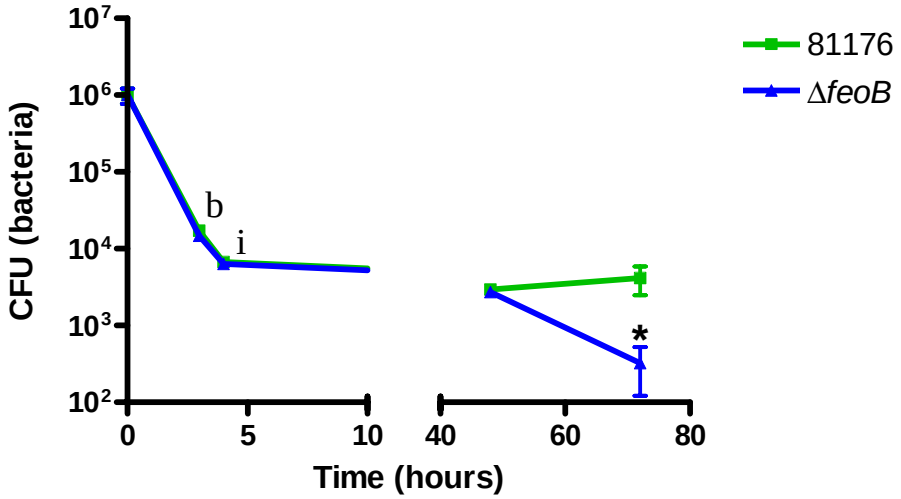

125000
100000
75000
50000
25000
0
Average CPM/OD
periplasmic
spheroplastic
periplasmic
spheroplastic
fractions
NCTC
ΔfeoB
*
*

Figure 7 A) Intracellular survival, binding and invasion efficiencies of *C. jejuni* 81-176 wild-type strain and the *feoB* mutant within INT-407 epithelial cells. **B)** Intracellular survival, binding and invasion effiecies of *C. jejuni* 81-176 wild-type strain and the *feoB* mutant within IPEC-1 epithelial cells. Epithelial cells were infected with the wild type strain and Δ*feoB* mutant at a multiplicity of infection 10:1 (number of bacteria per eukayotic cell) and survival kinetics were analyzed over a 72 hour period. "b" represents the binding efficiency and was obtained by subtracting the number of invaded bacteria from the total number of bacteria recovered without gentamicin treatment. "i" represents the invasion efficiency and was calculated as the percentage of inoculum recovered after gentamicin treatment. The experiment was repeated thrice and the data represents the mean ± standard error. * indicates statistically significant difference at a 5 % level of significance, by Student *t* test, for the 72 hour time point.

Figure 7

A)


81176
ΔfeoB
CFU (bacteria)
Time (hours)
b
i
*

B)

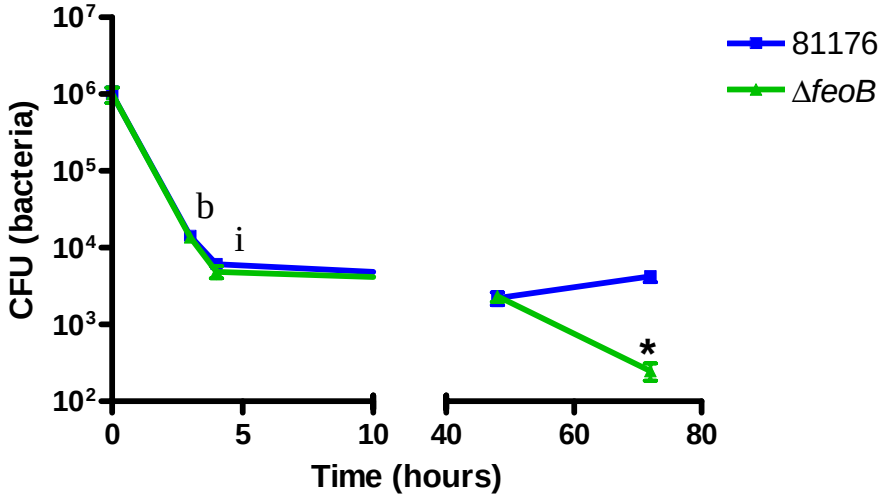

81176
ΔfeoB
CFU (bacteria)
Time (hours)
b
i
*

Figure 8: Non competitive chick colonization assay. Comparative colonization properties were tested in wild-type and *feoB* mutant strains of *C. jejuni* NCTC 11168, *C. jejuni* TGH 9011 and *C. jejuni* 81-176. Five birds per group were orally challenged with 10^4-10^5 cfu. The columns represent the means and the error bars indicate the standard errors. Non parametric Mann-Whitney Rank Sum test was used for statistical analysis. * indicates statistically significant difference at a 5 % level of significance.

Figure 8:

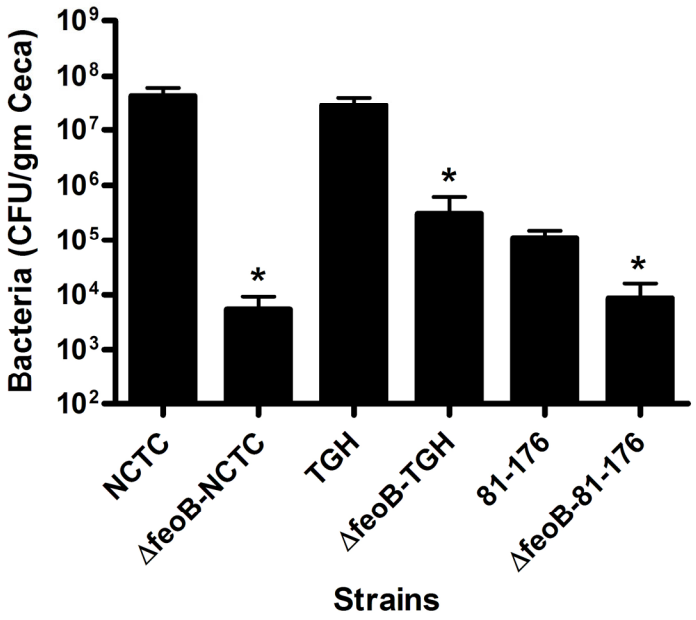

Bacteria (CFU/gm Ceca)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
*
*
*
NCTC
ΔfeoB-NCTC
TGH
ΔfeoB-TGH
81-176
ΔfeoB-81-176
Strains

Figure 9: Competitive colonization ability of *feoB* mutants of *C. jejuni* NCTC 11168 and *C. jejuni* TGH 9011. A 1:1 mixed inocula of the wild-type and *feoB* mutant strains were orally inoculated into five birds. The *in vivo* competitive index is the ratio of the output mutant to the wild-type recovery divided by the ratio of the input mutant to wild type strain. The data represents the mean ± standard errors. * indicates statistical significance ($p<0.0001$).

Figure 9:

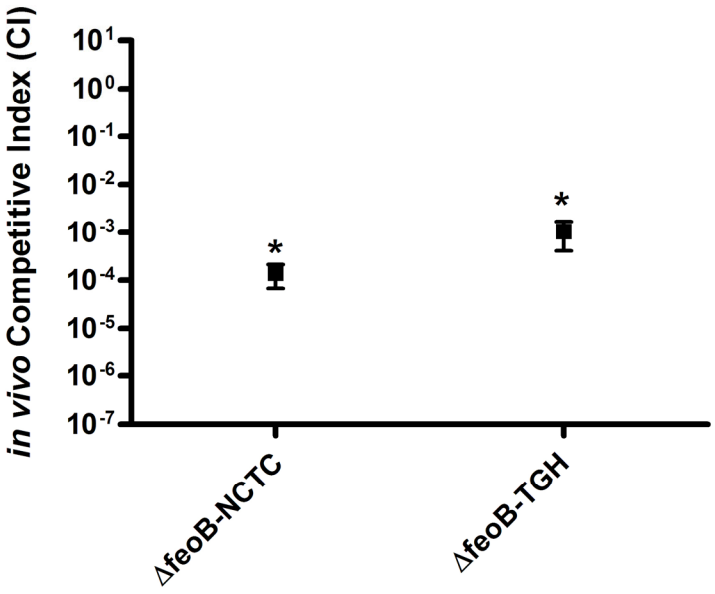
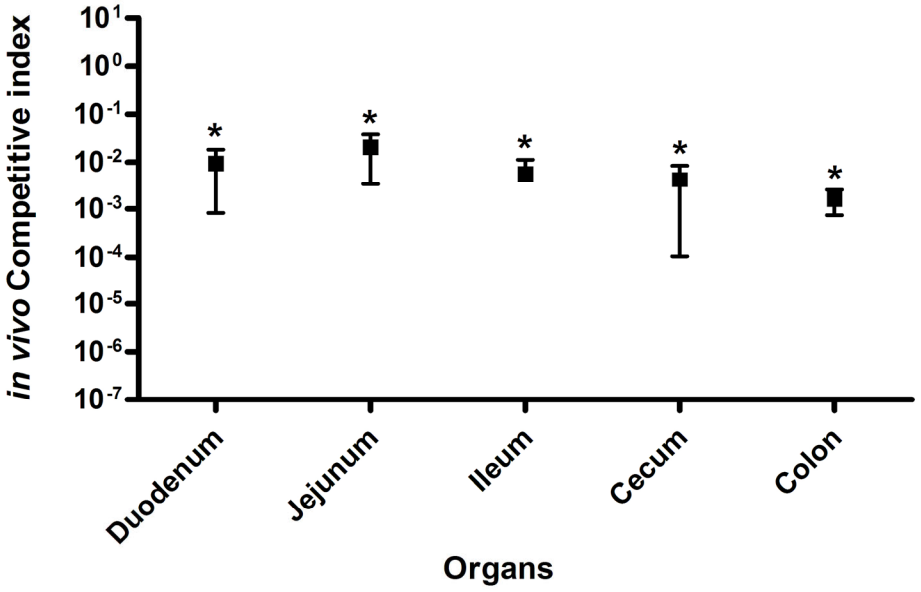

in vivo Competitive Index (CI)
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
*
ΔfeoB-NCTC
ΔfeoB-TGH

Figure 10: Colostrum-deprived neonatal piglet competitive colonization assay.

A) The competitive colonization ability of the *C. jejuni* NCTC 11168 wild-type strain and its *feoB* mutant was assessed. Three piglets were orally challenged with a 1:1 mixed inocula of the wild-type and mutant strain. Seventy-two hours post inoculation, the pigs were euthanized and the intestinal segments- duodenum, jejunum, ileum, cecum and colon were collected. The intestinal contents were checked for the wild-type and *feoB* mutant titers. The CI was calculated as the ratio of the output mutant to the wild-type strain recovered divided by the ratio of the input mutant to the wild-type strain inoculated. The data represents the mean ± standard errors. * indicates statistical significance ($p<0.0001$). **B)** The competitive colonization ability of *C. jejuni* TGH 9011 wild-type strain and its *feoB* mutant was assessed in three piglets. The data represents the mean ± standard errors. * indicates statistical significance ($p<0.0001$). **C)** The competitive colonization ability of *C. jejuni* 81-176 wild-type strain and its *feoB* mutant was assessed in two piglets.

Figure 10:

A)


in vivo Competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
Colon
Organs

B)

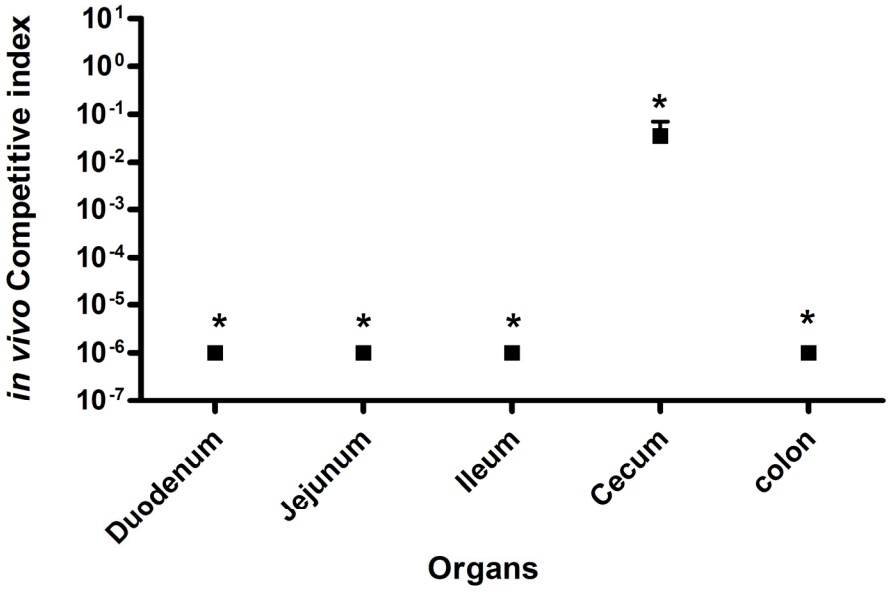

in vivo Competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

Figure 10:

C)

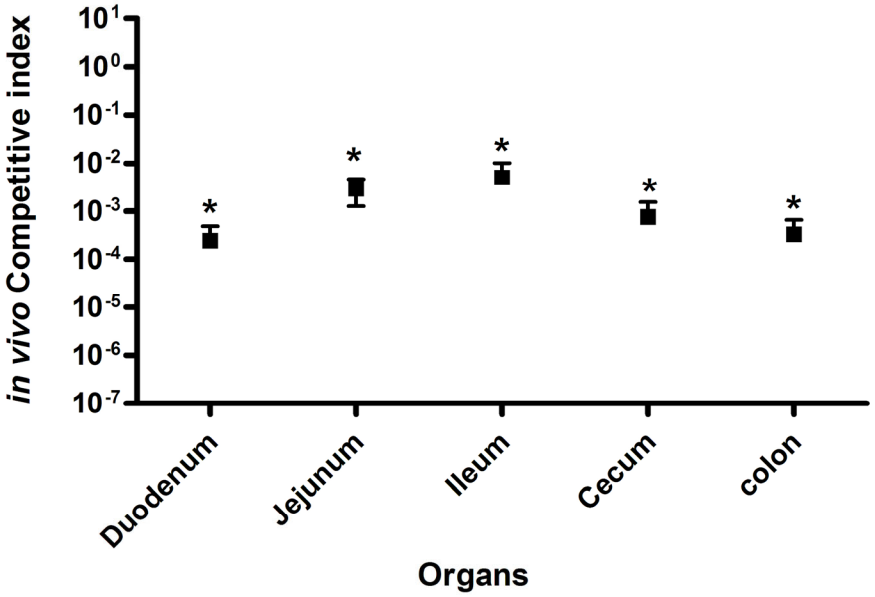

in vivo Competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
*
*
*
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

Figure 11: Rabbit *in vivo* competitive colonization and *in vitro* competitive growth assay. The *in vivo* phenotype of *feoB* mutant was assessed in a 1:1 competition assay. The *feoB* mutant was mixed with the wild-type strain and inoculated into four ileal loops. At 48 hours post infection, the loop contents were checked for the wild-type strain and mutant titers. The CI was calculated as the ratio of the output mutant to the wild-type strain recovered divided by the ratio of the input mutant to the wild type strain inoculated. The i*n vitro* competition growth index was performed in triplicate. The *in vitro* CI represents the ratio of the *feoB* mutant to the wild-type *C. jejuni* NCTC 11168 in MH at early stationary phase. The data represents the mean ± standard errors. * indicates statistical significance at a 5 % level of significance (p=0.0176).

Figure 11:

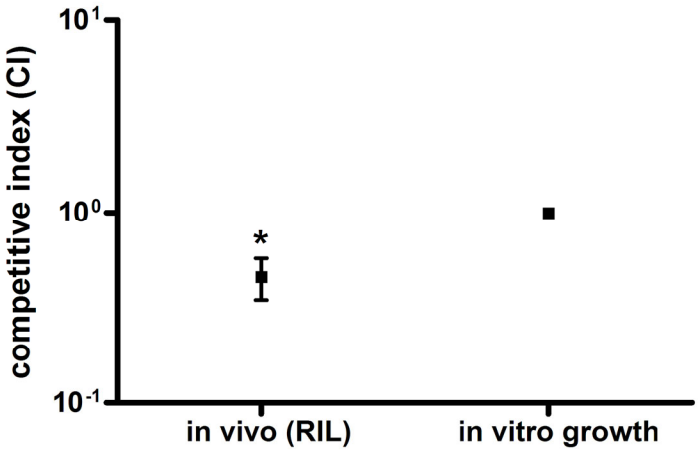
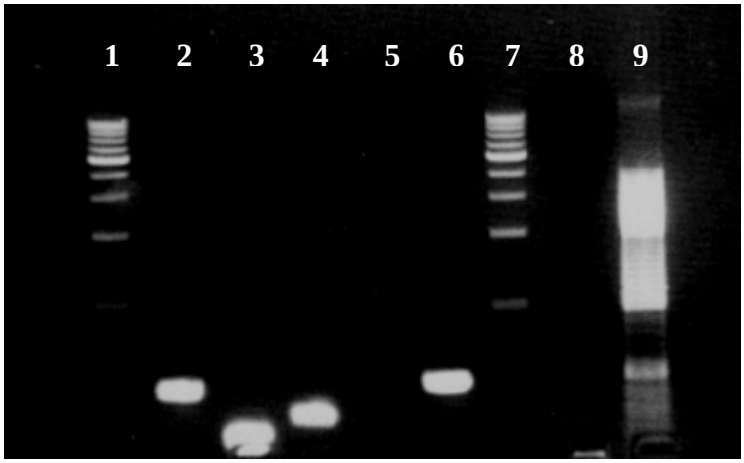
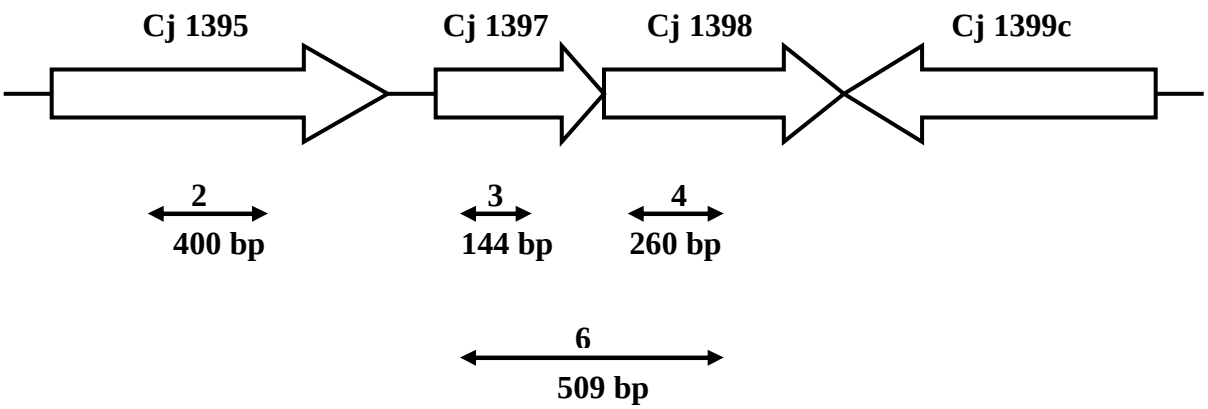


Figure 12: Cj1397-Cj1398 operon mapping by RT-PCR. The template RNA was purified from mid-log-phase bacteria grown in iron-limited medium MEMα. Predicted RT-PCR fragments with gene names are shown at the bottom. The gel lanes match the RT-PCR fragment labels. Lanes 1 and 7 corresponded to the 1 Kb ladder, lane 9 was a 100 bp ladder. Lane 8 was no RNA template control.

Figure 12:


1 2 3 4 5 6 7 8 9


Cj 1395
Cj 1397
Cj 1398
Cj 1399c
2
400 bp
3
144 bp
4
260 bp
6
509 bp

Chapter IV

Role Of The TonB Systems Of *Campylobacter Jejuni* In Iron Acquisition And Colonization

Hemant Naikare, Kiran Palyada, Alain Stintzi

Department of Veterinary Pathobiology

Oklahoma State University

Stillwater OK 74078

Abstract

We have characterized the TonB systems from three strains of *C. jejuni*, *C. jejuni* NCTC 11168, *C. jejuni* ATCC 43431 (TGH 9011) and *C. jejuni* 81-176, by defined mutagenesis, and assessed their role in the gastrointestinal colonization of day-old chicks and colostrum-deprived neonatal piglets. Analysis of the sequenced isolate, *C. jejuni* NCTC 11168 genome, revealed the presence of three *tonB* genes, *tonB1*, *tonB2* and *tonB3*. We demonstrated that the three TonB systems performed specific as well as redundant functions. Bioassays indicated that TonB3 specifically mediated the utilization of the siderophores enterobactin and lysine CAM, in *C. jejuni* NCTC 11168. The *tonB2* and *tonB3* single mutants were not affected in hemin and hemoglobin utilization, whereas the *tonB2tonB3* double mutant was found to be completely impaired in the utilization of the above mentioned sources. Interestingly, the single *tonB3* mutant of *C. jejuni* TGH 9011 is unable to utilize enterobactin, hemin and hemoglobin, whereas the single *tonB2* mutant is unaffected in utilization of these iron sources as compared to the wild-type strain. The *tonB2* mutant of *C. jejuni* 81-176 is completely impaired in the utilization of hemin, hemoglobin and lysine CAM. The construction of a triple *tonB* mutant in *C. jejuni* NCTC 11168 was not successful due to the paucity of alternative genetic tools available for *C. jejuni*. Interestingly, the $^{55}Fe^{3+}$ - enterobactin uptake assays further corroborated that the single *tonB* mutant was significantly compromised as compared to the wild-type strain. As expected, the double *tonB* mutants, *tonB1tonB3* and *tonB2tonB3* were also

significantly affected in $^{55}Fe^{3+}$ - enterobactin transport as compared to the wild-type strain. The TonB systems of *C. jejuni* were found to be not involved in the adhesion and invasion of the INT-407 human intestinal epithelial cells. We assessed the role of TonB systems in colonization of the gastrointestinal tract using the avian and piglet colonization models. All the single and double *tonB* mutants were significantly affected in their ability to colonize the gastrointestinal tract of both animal models. The *tonB1tonB3* double mutant was the most significantly affected, of all the combinations of mutants studied. Interestingly, the *tonB2tonB3* double mutants and the *tonB1tonB2* double mutants were more compromised than the single *tonB1* or single *tonB3* mutants in colonization, further suggestive of the additive role of *tonB2* in iron acquisition. The above *in vivo* colonization phenotype was found to be the same when wild-type and mutants were assessed for competitive and non competitive colonization of the chicken ceca. Overall, these results indicate that TonB mediated iron acquisition in *C. jejuni* is an important colonization determinant and likely contributes towards the pathogenesis and establishment of infection.

Introduction

Iron is an essential nutrient for almost all pathogenic bacteria. Although iron is one of the most abundant elements on earth, its availability within the host is extremely limited due to its poor solubility. The availability of free iron at physiological pH within the host is 10^{-18} M, whereas, the requirements of all microbes is approximately 10^{-8} M (35). Within the host, iron is usually either bound to proteins such as lactoferrin, transferrin and ferritin, or iron is present in hemoglobin (28, 45). The pathogenic bacteria usually employ two major mechanisms to acquire iron from host sources, which include high-affinity iron chelator (siderophore) mediated iron uptake and the uptake from host iron binding protein (transferrin, lactoferrin) or heme uptake via specific receptors (1, 44). The transport of ferric-siderophores or heme compounds in several gram-negative bacteria has been demonstrated to require specific outer membrane receptors and is dependent upon the TonB ExbB ExbD complex (34). The TonB transduces the energy from the cytoplasmic membrane to the outer membrane receptors for the transport of iron compounds (5, 21). The TonB dependent iron transport system has been found to contribute in the virulence of several gram-negative pathogens such as *Shigella dysenteriae, Vibrio cholerae, Vibrio anguillarium, Pseudomonas aeruginosa, Salmonella enterica* serovar typhimurium*, Salmonella enterica* serovar typhi and *Haemophilus influenzae (12, 17, 18, 36, 39-41)*. The food-borne pathogen *Campylobacter jejuni*, is a major cause of bacterial enteritis in humans world-wide (42, 43). With the availability of the complete genome sequence of *C. jejuni* NCTC 11168 in 2000, it has led to newer opportunities in better understanding of the molecular mechanism of pathogenesis. Iron acquisition has been considered as a potential colonization and virulence determinant in

C. jejuni (30). Recently, the complex response of *C. jejuni* to the availability of iron in the environment was characterized (16, 29). The *C. jejuni* genome lacks the genes responsible for biosynthesis of siderophores (30). Although a few strains of *C. jejuni* were identified to synthesize siderophores, they have not been fully characterized (10). It is not known whether *C. jejuni* NCTC 11168 produces siderophores. Strikingly, the genome has genes responsible for enterobactin uptake (30, 43). Besides, it has been recently reported that cfrA, the outer membrane receptor functions in the transport of ferric-enterobactin in *C. jejuni* NCTC 11168 (29). However, the *cfrA* gene is not present in all strains of *C. jejuni* (43). It has also been documented that the periplasmic protein complex, ceuBCDE, mediates the uptake of enterobactin in *C. jejuni*. The chuABCD system mediates the uptake of heme in *C. jejuni* (19, 42, 43). Analysis of the *C. jejuni* NCTC 11168 genome reveals the presence of three possible TonB systems. TonB1 is 248 amino acids in length and is the largest of the three TonB systems (30). Both the TonB2 and TonB3 are 227 amino acids in length and have 41% identity. Identity between TonB1 and TonB3 is 19% and that between TonB1 and TonB2 is 23%. *E. coli* TonB and *C. jejuni* TonB1 have 22% identity, *E. coli* TonB and *C. jejuni* TonB2 have 26% identity, while *E. coli* TonB and *C. jejuni* TonB3 have 28% identity (30).

It is likely that the transport of ferric enterobactin, hemin and other iron compounds is TonB dependent in *C. jejuni*. However, the role of *tonB* genes in iron transport of *C. jejuni* has not yet been experimentally demonstrated. To assess the role of these TonBs in iron uptake, we constructed a series of single and double *tonB* mutants in *C. jejuni* NCTC 11168. The effect of these mutations was tested by *in vitro* and *in vivo* assays using chick colonization model and colostrum-deprived neonatal piglet model. We also

characterized the TonB2 system from *C. jejuni* 81-176 and the TonB2 and TonB3 systems from *C. jejuni* TGH 9011 by *in vitro* assays.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial plasmids and strains used in this study are listed in Tables 1 and 2 respectively. Luria-Bertani (LB) broth or LB agar plates were used to routinely culture *E. coli* DH5α at 37˚C, aerobically. Media supplemented with kanamycin (30 μg/ml) and/or chloramphenicol (20 μg/ml) were used to grow strains containing plasmids. *C. jejuni* 81176 and *C. jejuni* ATCC 43431 (TGH 9011) were obtained from Dr Pickett and the American Type Culture Collection, respectively. *C. jejuni* NCTC 11168 was obtained from the National Collection of Type Cultures. Routine culturing of these strains was done at 37˚C in a MACS-VA500 microaerophilic workstation (Don Whitley, West Yorkshire, England) under 83 % N_2, 4 % H_2, 8 % O_2 and 5 % CO_2 on Mueller Hinton (MH) agar plates, MH medium or minimum essential medium (MEMα, Invitrogen). Kanamycin (30 μg/ml) or chloramphenicol (20 μg/ml) or both were added as required. The *C. jejuni* strains were tested for motility on 0.4 % MH agar plates before performing any *in vitro* cell culture studies or *in vivo* animal experiments. For the *in vitro* growth assays, the *C. jejuni* wild type and mutant strains were grown in MH biphasic medium or MEMα or MEMα supplemented with 40 μM $FeSO_4$ at 37˚C in the microaerophilic workstation.

Cell Culture

Human INT-407 embryonic intestinal cells (acquired from the American Type Culture Collection) were cultured in minimum essential medium α (MEM α, Invitrogen), supplemented with 10 % fetal bovine serum (Invitrogen). Cells were grown in an incubator at 37˚C under 5 % CO_2.

Binding and invasion assays

These assays were performed as described previously (33). Briefly, the binding assay was carried out by co-incubating mid-log-phase *C. jejuni* cells grown in biphasic MH media with 24 hour grown, semi-confluent INT-407 cells (about 10^5 cells per well) at a multiplicity of infection (MOI) of 10:1 (10 bacteria per eukaryotic cell). Three hours post incubation at 37˚C, in the presence of 5 % CO_2, the cell growth medium was removed, and the monolayer was washed thrice with Hanks balanced salt solution (HBBS). Subsequently, infected cells were lysed with 0.1 % Triton X-100, at room temperature, for 30 minutes. Then the cell lysates were serially diluted and plated on MH agar plates to enumerate the number of bacteria bound to the eukaryotic cells and internalized within the eukaryotic cells.

The invasion assay was performed similar to the binding assay, except that the infected cells were further incubated for an additional one hour in fresh medium with 250 μg of gentamicin per ml, to kill the extracellular bacteria. Subsequently, the cells were washed thrice with Hanks balanced salt solution, and were then lysed using 0.1 % Triton X-100 for 30 minutes at room temperature. Cell lysates were serially diluted and plated on MH

agar plates to enumerate the number of bacteria that invaded within the eukaryotic cells. The invasion efficiency was expressed as the percentage of inoculum recovered after gentamicin treatment, whereas the binding efficiency was expressed by subtracting the number of invaded bacteria from the number of bacteria recovered from cells not subjected to gentamicin treatment. The results shown for the binding and invasion assays represent the means of three independent experiments ± standard error.

Construction of the *C. jejuni tonB* mutants

Single *tonB* mutant construction

Single *tonB1, tonB2* and *tonB3* mutants of *C. jejuni* NCTC 11168 were constructed using similar inactivation strategy. *C. jejuni* NCTC 11168 chromosomal DNA was prepared using the Wizard genomic kit (Invitrogen). The *tonB1* gene was PCR amplified using the primers TonB11 and TonB12 (each primer containing a *Bgl*II site). The resulting 1623bp PCR product was digested with *Bgl*II, and ligated to the *Bam*HI restricted suicidal vector pUC19, yielding pAS16. An inverse PCR was performed to delete 665bp within the *tonB1* gene with TonB13 and TonB14 primers (each primer containing a *Bam*HI restriction site). The PCR product was cut with *Bam*HI, self ligated and introduced into *E. coli* DH5α, yielding the plasmid pAS20. A *Bam*HI restricted 1.4 Kb Km^r cassette from pILL600 plasmid was then cloned into the previously *Bam*HI restricted pAS20, yielding pAS23. The orientation of the Km^r cassette was confirmed by sequencing, to be in the same direction as the *tonB1* gene and then transformed into *C. jejuni* NCTC 11168 by natural transformation to generate the *tonB*1 mutant by allelic exchange. The natural transformation was performed as follows: *C. jejuni* wild-type cells were grown in MH

biphasic medium to mid-log-phase. Lawns were made with 50 µl of the cultured *C. jejuni* cells on MH plates and allowed to grow at 37ºC in the microaerophilic workstation for 12 hours. Then 1-1.5 µg of final construct plasmid DNA was spotted on the lawns and were incubated for an additional 12 hours at 37ºC in the microaerophilic workstation to permit the natural transformation of the wild-type cells. Thereafter, the lawns were harvested with 200 µl of MH broth, resuspended and spread onto MH plates containing kanamycin (30µg/ml) for the selection of transformants. The plates were incubated for 3-5 days at 37ºC in the microaerophilic workstation to allow the growth of transformants. The transformants were further confirmed for antibiotic resistance by checking for growth on antibiotic containing plates. Subsequently, the chromosomal DNA was extracted from the mutant clones and checked by PCR for confirmation of homologous double recombination events using a combination of primers that amplified the Km^r and *tonB* genes. The *tonB2* (AS240) and *tonB3* (AS239) mutants were constructed following a similar strategy as the one that was used for constructing *C. jejuni* NCTC 11168 *tonB1* mutant. Briefly, a 1668bp DNA fragment containing the *tonB2* gene was amplified by PCR using primers TonB21 and TonB22 (each primer contains a *Bgl*II site). The PCR product was digested with *Bgl*II and subsequently cloned into *Bam*HI restricted pUC19 to obtain the plasmid pAS17. A 510bp deletion was made within the *tonB2* gene (yielding pAS24) by inverse PCR using the primers TonB23 and TonB24 (each primer contains a *Bam*HI site). Subsequently the 0.8 Kb Cm^r cassette from pRY111 was introduced into the created *Bam*HI site of the *tonB2* gene, yielding the final construction pAS176. The orientation of the Cm^r cassette was confirmed by sequencing, to be in the same direction as the *tonB2* gene and then transformed into *C. jejuni* NCTC

11168 by natural transformation to generate the *tonB*2 mutant by allelic exchange. The natural transformation was performed as described earlier and transformants were selected on MH plates containing chloramphenicol (20µg/ml). For the construction of the *tonB3* mutant, a 1.6 Kb DNA fragment containing the *tonB3* gene was obtained by PCR using the primers TonB31 and TonB32 (each primer contains a *Bgl*II site). This PCR product was digested with *Bgl*II and cloned into the *Bam*HI restricted pUC19 vector, yielding pAS41. A 500bp deletion was created within the *tonB3* gene by inverse PCR using the primers TonB33 and TonB34 (pAS42) (each primer contains a *Bam*HI site). Finally, the 1.4 Kb Km^r cassette from pILL600 was introduced into the created *Bam*HI site of the *tonB3* gene, yielding the final construct pAS356. A similar strategy was adopted to make single mutants in the *tonB* genes from the non-sequenced clinical isolates of *C. jejuni*, *C. jejuni* ATCC 43431 (TGH 9011) and *C. jejuni* 81-176 using the final constructs described above. The single *tonB2* mutants of *C. jejuni* TGH 9011 (AS183) and *C. jejuni* 81-176 (AS252) were constructed using the final construct pAS176 and the single *tonB3* mutant of *C. jejuni* TGH 9011 (AS235) was constructed using the final construct pAS356. The primers and plasmids used in these constructions are listed in Tables 1, 2 and 3.

Double *tonB* mutant construction

The double *tonB* mutants of *C. jejuni* NCTC 11168 Δ*tonB1tonB2* (AS241), Δ*tonB2tonB3* (AS242) and Δ*tonB1tonB3* (AS355) were constructed using the final constructs described above and pAS348. The pAS348 (pUC19 carrying Δ*tonB1::*Cm^r) final construct was

created by cloning the *Bam*HI restricted 0.8 Kb *Cm*^{r} resistance gene into the previously *Bam*HI restricted pAS20 (pUC19 carrying Δ*tonB1*). The single *tonB* mutant strains were used as the recipient strains and were transformed using the final constructs by the similar allelic strategy. The double *tonB* mutant Δ*tonB2tonB3* (AS356) of *C. jejuni* TGH 9011 was constructed using the AS235 as the recipient strain and the pAS176 final construct by the same allelic exchange procedure. The primers and plasmids used in these constructions are listed in Tables 1, 2 and 3.

$^{55}Fe^{3+}$-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 the uptake buffer to an OD_{600}= 0.6 (equivalent to 10^9 bacteria per ml) and kept on ice. The uptake buffer was composed of 5 g/L Na_2HPO_4, 5 g/L KH_2PO_4, 1.18 g/L NH_4Cl, 0.089 g/L Na_2SO_4, 0.042 g/L $MgCl_2.6H_2O$ and 10 g/L casamino acids. Then, the resuspended cells were allowed to equilibrate at 37˚C for 10 minutes before performing the uptake assay. The $^{55}Fe^{3+}$-enterobactin complex used as the iron source was prepared as follows: 10 µl of 10 mM enterobactin was added to 5 µl of $^{55}FeCl_3$ (3.6 mM stock solution), followed by the addition of 182.18 µl water and 2.88 µl of $FeCl_3$ (0.025 M) to form the complex. This complex was incubated at room temperature for 30 minutes, followed by the addition of 100 µl of sodium phosphate buffer (0.5 M) and 700 µl of double distilled water to make the volume to 1000 µl. This final volume was centrifuged at 13000 rpm for 10 minutes and the supernatant was collected. 100 µl of the supernatant was taken and diluted with 9.9 ml of the uptake buffer. This diluted $^{55}Fe^{3+}$-enterobactin complex was filtered twice

using a 0.45 μm nitrocellulose millipore filter. One ml of the filtered diluted $^{55}Fe^{3+}$-enterobactin complex was added to 10 ml of *C. jejuni* cells (achieving final concentration of 0.018 μM). The uptake assay was performed using Millipore filtering assembly (Millipore Corporation, Ledford, MS). One ml aliquots 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 the 0.45 μm nitrocellulose millipore filter and vacuum filtered. Five ml of 0.1 M citric acid buffer was used to wash away the unlabeled complex and the filters were allowed to dry and were then immersed in 5 ml of the scintillation cocktail (Scintiverse, USA), vortexed and measured for the scintillation counts in the Beckmann LS5000 TD scintillation counter. Activity as counts per minute (CPM) were corrected for background. To demonstrate whether uptake was proton dependent, 33 μM of the protonophore carbonyl-cyanide-m-chlorphenyl hydrazone (CCCP) was added to the cells 3 minutes after uptake was initiated with another batch of cells.

Bioassay

The ability of the *C. jejuni* wild-type and mutant strains to utilize the different iron sources were tested on iron-limited assay plates as described previously (3, 10, 29). 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. The cells (1 ml) were seeded into 25 ml of MH medium containing 20 μM DFO (desferriferrioxamine), poured into Petri plates and allowed to solidify. Sterile disc papers containing 10 μl of 10 mM enterobactin, hemin, hemoglobin and other iron siderophore

sources were laid on the surface of the plate and the zone of growth promotion was measured after 24 hours of incubation at 37˚C under microaerophilic conditions.

Sequencing of *tonB2* gene from *C. jejuni* 81-176 strains

The *tonB2* gene was PCR amplified from *C. jejuni* 81-176 strains using the primers TonB21 and TonB22 and high fidelity *Pfx* polymerase enzyme (Invitrogen). The PCR products were sequenced at the DNA core facility (Oklahoma State University) using primers TonB21 and TonB22. The sequences were compared to the *tonB2* gene sequence from *C. jejuni* NCTC 11168 and were aligned using the Clustal W alignment software (Biology work bench, San Diego, CA).

In vivo colonization studies

Chick colonization assays

The two kinds of chick colonization experiments were conducted: non-competitive and competitive colonization assays.

Non-Competitive Chick colonization

On arrival of the one day old specific pathogen free chicks (Tyson farms, Arkansas), cloacal swabs were taken to make sure that they were *Campylobacter* free. The chicks were housed at 25˚C, and a brooder 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 grown under microaerophilic

conditions in an MH biphasic medium. Mid-log-phase bacteria were harvested and resuspended in PBS buffer. An inoculum of 0.25 ml of a bacterial suspension (which contained approximately 10^4 to 10^5 viable bacteria) was fed orally to each bird. The negative control was an uninoculated group of birds. Five birds per group were inoculated separately with the wild-type strain or mutant. The colonization potential was determined four days post challenge, as per standard procedures described previously (29). Following euthanasia, the ceca were collected; the contents were homogenized and examined for viable counts by plating on plates of *Campylobacter* agar base (Oxoid CM935) along with the *Campylobacter* selective Karmali antimicrobial supplements (Oxoid SR167E). Post incubation at 37˚C for 72 hours, the titer was determined and expressed as cfu per gram of ceca. Statistical analysis at 5% level of significance was done using the non-parametric Mann-Whitney Rank sum test.

Competitive chick colonization

The competitive colonization studies were done using five chicks per group. Each bird was inoculated with 0.5 ml of bacterial suspension containing a 1:1 mixture of wild-type and *tonB* mutant strains. Approximately 10^5 viable bacteria each, of the wild-type and of the mutant, were present in the mixed inoculum, which was confirmed by plating serial dilutions of the mixed culture on MH agar with and without kanamycin (30 μg/ml) and/or chloramphenicol (20 μg/ml). Four days post inoculation, the ceca were collected as described above. Homogenization of the cecal contents was followed by serial dilutions in PBS buffer, and plating on *Campylobacter* agar base (Oxoid CM935), enhanced with the *Campylobacter* selective Karmali supplement (Oxoid SR167E) and on Karmali agar

plate containing kanamycin (30 μg/ml) and/or chloramphenicol (20 μg/ml). The 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 titer was calculated by subtracting the number of mutants from the total number of bacteria recovered on Karmali agar plates without kanamycin and/or chloramphenicol. 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 at a 5 % level of significance was done using a single sample Student *t* test.

Piglet Colonization studies

Colostrum-deprived neonatal piglets were acquired from the Oklahoma State University swine farm, Oklahoma. After farrowing, the piglets were wiped off with Betadine and transported to the Laboratory Animal Research facility, OSU, Oklahoma. The uninoculated control and the experimental piglets were housed in separate pens. Approximately 80-100 ml of protein-rich and antibody-free reconstituted commercial replacer milk ration (Sav-A-Calf, Wisconsin) was fed to the piglets, four times daily.

Upon arrival, rectal swabs were taken to ensure that the piglets were *Campylobacter* free. Prior to challenge with the mixed inocula, milk feeding was withheld for 2 hours. *C. jejuni* wild-type NCTC 11168 strain and *tonB* mutants were grown in biphasic MH medium to mid-log-phase, after which, they were centrifuged and resuspended in Similac milk (commercial milk formula) to an OD_{600} of approximately 2.0 (corresponding to

~ 5 X 10^{10} CFU/ml). The mutant and wild-type strains were mixed in a 1:1 ratio (5 ml each). The mixed inocula were fed to two piglets orally. The bacterial counts in the mixed inocula was confirmed by plating serial dilutions on MH agar with and without kanamycin (30 μg/ml) or chloramphenicol (20 μg/ml) for single *tonB* mutants, and with and without kanamycin (30 μg/ml) and chloramphenicol (20 μg/ml) for double *tonB* mutants. Milk without any bacteria was fed to control piglets.

Piglets were observed daily for clinical signs such as fecal consistency, diarrhea and general health. The presence of blood in the feces was determined by using EZ detection kit (Biomerica). Seventy two hours after inoculation, the piglets were anesthetized with 0.25 ml/kg of ketamine (100 mg/ml) and 0.125 ml/kg of xylazine (20 mg/ml) (intramuscular), following which, they were euthanised with 0.25 ml/kg of Beuthenesia-D (intracardiac). Following necropsy, the intestinal segments duodenum, jejunum, ileum, cecum and colon were recovered. Gross lesions were examined in the piglets inoculated with the wild type-strain alone. The contents and the mucous layer of the intestinal segments were collected and homogenized in 5 ml of PBS buffer. The bacteria recovered from each organ were serially diluted and plated on *Campylobacter* selective media with and without antibiotics as described previously in the chick competitive colonization assay. The competitive index for each organ was computed as the ratio of the output mutant to the wild-type strain recovered divided by the ratio of the input mutant to the wild type-strain inoculated. A single sample Student *t* test was used to statistically analyze the data at a 5% level of significance.

Results and Discussion

Construction of *tonB* mutants

The genome sequence of *C. jejuni* NCTC 11168 reveals the presence of three *tonB* genes, *tonB1*, *tonB2* and *tonB3* (30). We hypothesize that the multiple TonB systems in *C. jejuni* have specific and redundant functions. Multiple TonB systems have been identified in other Gram-negative bacteria such as *Vibrio cholerae*, *Vibrio anguillarium*, *Pseudomonas aeruginosa* and *Haemophilus influenzae* (12, 24, 38, 39).

In order to determine the role of the three TonB systems of *C. jejuni* in iron acquisition, we mutated the *tonB1*, *tonB2*, and *tonB3* genes in *C. jejuni* NCTC 11168 by allelic exchange strategy. In order to study the role of TonB in cellular invasion and because the sequenced strain *C. jejuni* NCTC 11168 is poorly invasive (33), we attempted to mutate these genes in two other highly invasive clinical isolates, *C. jejuni* ATCC 43431 (TGH 9011) and *C. jejuni* 81-176 (32, 33). Although these isolates have not been sequenced, they have been recently compared by genome-wide analysis with the *C. jejuni* NCTC 11168 (9, 22, 33). Although all the three *tonB* genes were reported to be present in the *C. jejuni* TGH 9011 (which is also the Penner serotype 0:3 reference strain), we were unable to mutate *tonB1* using the same suicide vector as the one constructed to knock-out the *tonB1* gene of *C. jejuni* NCTC 11168. The *C. jejuni* 81-176 is a human isolate and has been documented to be a pathogenic, highly invasive strain (32, 33). We were successful in making a *tonB2* mutant in *C. jejuni* 81-176. The inability to make *tonB1* and *tonB3* mutants was in agreement with the findings of Leonard *et al* (22) who had demonstrated that in the *C. jejuni* 81-176 genome, the *tonB1* and *tonB3* genes were absent. In order to

elucidate the relative functions of these *tonB* genes in *C. jejuni*, we also constructed the double mutants of *tonB*s. Figure 1A and 1B are representative PCR confirmation of the mutant constructions. Attempts to construct a triple *tonB* mutant lacking all the three known TonB systems were not successful with the available genetic tools, thus impairing the complete characterization of the TonB systems. Nevertheless, the construction of all the possible series of single and double TonB knock-outs allowed the characterization of their roles in iron acquisition and colonization of the gastrointestinal tract of the host.

Growth characteristics of *tonB* mutants *in vitro*

In order to determine whether the *C. jejuni tonB* mutants were affected in their ability to grow *in vitro*, we assessed the growth of the wild-type *C. jejuni* strain and *tonB* mutants in MH medium and in MEMα with or without 40 μM $FeSO_4$. Growth in MH medium was tested for all the three *C. jejuni* isolates and their corresponding *tonB* mutants. There was no statistically significant difference in the growth rate of the wild type and the *tonB* mutants of *C. jejuni* NCTC 11168 (Figure 2 A, B), *C. jejuni* TGH 9011 (Figure 3A) or *C. jejuni* 81-176 (Figure 3B). The MH medium is not an iron free medium and the exact amount of iron in MH medium is not known, so, the growth studies with MH medium represent the phenotype of the wild-type and *tonB* mutants in iron containing medium.

Further, we examined the *in vitro* growth of *C. jejuni* NCTC 11168 wild-type strain and its *tonB* mutants in iron defined medium (Figure 4A, 4B). The growth rate of the wild-type and *tonB* mutants were compared in the absence and presence of iron. There was a significant increase ($p < 0.05$) in the growth rate in the presence of iron as compared to the growth rate in the absence of iron for the wild type strain as well as the

mutants. These results indicate that the *tonB* mutant and the wild type strains exhibited a slower growth rate in iron limited medium. Similar results were observed by Field *et al* (10). We further compared the difference in the growth rates of the wild-type strain with the *tonB* mutants in the presence and absence of iron. At a 5% level of significance, there was a significant difference ($p<0.05$) in the growth rate of the wild-type strain with its *ton*B mutants. Also as expected, there was a significant difference in the growth rates of the wild-type strain, the *tonB2tonB3* double mutant ($p<0.04$) and the *tonB1tonB3* double mutants ($p<0.03$). The *tonB3* single mutant and the two double mutants, *tonB2tonB3* and *tonB1tonB3,* grew at a significantly slower rate than the wild-type strain. This data indicates that the *tonB3* mutants were significantly affected in the growth rates as compared to the wild type strain when grown in the iron limited medium. Therefore, this *in vitro* growth defect suggests that the presence and absence of iron in the growth medium influences the growth pattern of the *C. jejuni* strains and that the *tonB* genes are likely to be iron regulated.

The *tonB* mutants are not affected in binding and invasion of INT-407 cells

In order to determine the effect of the *tonB* mutation on binding and invasion of the INT-407 eukaryotic cells, we performed binding and invasion assays with all three *C. jejuni* isolates, which have been well characterized for their invasion ability. *C. jejuni* NCTC 11168 is a poor invader of INT-407 cells, whereas *C. jejuni* TGH 9011 and *C. jejuni* 81-176 are highly invasive isolates. The ability of *C. jejuni* to invade and survive within the eukaryotic cell has been shown to be a virulence determinant (7, 31). The intracellular growth within epithelial cells has been shown to be essential for

virulence in the Gram-negative bacterium *S. typhi* (23). Table 4 shows that none of the *tonB* mutants were significantly affected ($p>0.05$) in their binding and invasion efficiencies. In this study, we did not determine if the TonBs played a role in intracellular survival. It has been shown in *Salmonella enterica serovar typhi*, that the growth of the *tonB* mutant in the human monocyte cells was significantly restricted compared to the wild-type strain (12). A similar study with *Shigella dysenteriae tonB* mutant had demonstrated that invasion efficiency of the mutant was not affected, however, the intracellular survival and spread was significantly affected (36). Therefore, intracellular survival experiments with *C. jejuni tonB* mutants might shed light on the role on TonBs in the survival within the host cells.

The *tonB* mutants were affected in utilization of iron sources in growth promotion assays

In order to determine whether *tonB* mutants were defective in their ability to utilize siderophores and other iron compounds, we performed growth promotion assays (bioassays) as described in the materials and methods section. *C. jejuni* is known to colonize the enteric environments of its hosts (19) and, to survive inside the epithelial cells (31) and the macrophages (7). This facultative intracellular pathogen therefore has access to different potential sources of iron, although the exact sources which *C. jejuni* utilizes in the different environments is not clearly known. Whole *C. jejuni* NCTC 11168 genome does not contain any siderophore biosynthesis genes (30), however, it does possess the machinery for enterobactin uptake. Enterobactin is a powerful tris-catecholate siderophore synthesized by *E. coli* (8). It has been demonstrated that *C. jejuni* can utilize

the ferric-enterobactin complex for its growth and iron transport (29). We have also tested the *tonB* mutants for their ability to utilize another catecholate, lysine catecholamide (lysine CAM). The lysine CAM is structurally related to enterobactin such that it is a tetradentate chelator while enterobactin is a hexadentate chelator. Therefore, the lysine CAM provides four chelating atoms to coordinate iron, whereas the enterobactin provides six chelating atoms to fully coordinate the iron. Lysine CAM has been reported to be synthesized by *Azotabacter vinelandii* (20). The probable intracellular iron sources, hemin and hemoglobin were also screened for utilization by the *tonB* mutants. Therefore, in order to identify whether the multiple TonB systems in *C. jejuni* have any substrate specificity and whether they exhibit any redundancy in iron utilization from different sources, we studied their growth promotion ability. As seen in Tables 5 and 6, the single *tonB3* mutant of *C. jejuni* NCTC 11168 was defective in utilization of enterobactin, whereas the single *tonB1* and *tonB2* mutants were not affected. As expected, the double mutants of *tonB3, tonB2tonB3* and *tonB1tonB3* mutants also exhibited the same phenotype as the *tonB3* single mutant. Similar phenotype was observed with the lysine CAM siderophore utilization by *C. jejuni* NCTC 11168 *tonB* mutants.

The *C. jejuni* TGH 9011 and *C. jejuni* 81-176 wild-type strains were unable to utilize enterobactin (Table 6). The *C. jejuni* 81-176 was able to utilize lysine CAM siderophore, whereas *C. jejuni* TGH 9011 was found to be deficient in the utilization of lysine CAM siderophore. The *tonB2* mutant of *C. jejuni* 81-176 was significantly defective in the transport of lysine CAM. Earlier, Guerry *et al* had shown in *Campylobacter coli* that the hemoglobin and hemin uptake was TonB-dependent (13). The utilization of hemin and

hemoglobin by *C. jejuni* was suggested to be mediated through the chuABCD transport system (37). Pickett *et al* reported that *C. jejuni* 81-176 utilized hemin and hemoglobin (32). Based on our bioassay data, the single *tonB2* and *tonB3* mutants of *C. jejuni* NCTC 11168 were unaffected in the utilization of hemoglobin and hemin, whereas a double *tonB2tonB3* mutant was completely defective in hemin and hemoglobin transports. This is the first report in which the uptake of hemin and hemoglobin in *C. jejuni* has been experimentally demonstrated to be TonB dependent. The *tonB2* mutant of *C. jejuni* 81-176 was found to be defective in utilization of hemin and hemoglobin. Based on the Leonard *et al* study, the *C. jejuni* 81-176 genome has only a single *tonB2* gene (22). Therefore, as expected, the *tonB2* gene of *C. jejuni* 81-176 appears to mediate the transport of all the iron sources tested and is likely to be the only functional *tonB* gene in this isolate.

These data indicate that TonBs in *C. jejuni* have specific and redundant functions. The *tonB3* mutant, based on our bioassay result, is required specifically for enterobactin, whereas the TonB2 and TonB3 together transport hemin and hemoglobin. Specificity and redundancy in function of TonBs phenotypes has also been demonstrated in *V. cholerae* (38). In *Vibrio cholerae*, both the TonBs are involved in hemin transport whereas TonB2 specifically mediates schizokinen siderophore transport.

We had also tested the ability of the wild-type *C. jejuni* isolates to utilize siderophores and iron sources such as cepabactin, aerobactin, ferrichrome, rhodotuloric acid, transferrin and lactoferrin. None of these sources were utilized by any of the wild types of *C. jejuni*.

$^{55}Fe^{3+}$-enterobactin uptake assays

Transport of ferric-siderophores through outer membrane is proton dependent

The active transport of iron against its concentration gradient has been shown to be dependent on the proton motive force as well as ATP. This has been demonstrated in *E. coli* and other Gram-negative bacteria, but has not been experimentally proven in *C. jejuni* (1, 5, 6, 26). In order to test the proton dependency for ferric-siderophore uptake in *C. jejuni*, we used the protonophore carbonyl-cyanide-m-chlorphenyl hydrazone (CCCP). Enterobactin mediated iron uptake was monitored following treatment of the cells with CCCP. As shown in Figure 5A, in contrast to untreated cells, the CCCP treated cells were not able to transport ferric-enterobactin complex (0.018μM final concentration).

The *tonB* mutants were significantly affected in ferric-enterobactin uptake

In order to determine if the *tonB* mutants were defective in ferric-enterobactin uptake, we performed $^{55}Fe^{3+}$-enterobactin uptake assay and compared the uptake efficiencies of the *C. jejuni* NCTC 11168 wild-type strain and its *tonB* mutants. Cells grown in iron limited medium (MEMα), approximately 10^8 cells/ml were assessed over a 12 minute time-course following the addition of $^{55}Fe^{3+}$-enterobactin complex (0.018 μM final concentration of radiolabeled ferric-enterobactin). The uptake of the wild-type cells was compared with the uptake measured in the *tonB* mutant cells and was statistically analyzed by Student *t* test at a 5% level of significance. As shown in the Figure 5B and 5C, there was a statistically significant difference ($p<0.05$) in the ferric-enterobactin uptake for several of the single and double *tonB* mutants (except the *tonB2* mutant).

These data suggests the contribution of mainly the TonB1 and TonB3 towards the uptake of ferric-enterobactin in a short time course experiment. Both the ferric-enterobactin assay and the bioassay revealed similar findings. It is evident that while *tonB1* mutant is affected in its iron uptake kinetics, the expression of *tonB3* gene will permit the utilization of enterobactin for growth in iron restricted medium.

Notably, the bioassay and uptake assays confirmed the involvement of TonB3 in the utilization of enterobactin. The partial ferric-enterobactin uptake observed in the *tonB3* mutant corresponded to the uptake seen in CCCP treated cells (Figures 5A, 5B), which therefore represents the binding of enterobactin to the outer membrane receptor, and not actual transport. Binding of iron-free siderophores has been shown to be a common feature of siderophore outer membrane transporters of *Pseudomonas aeruginosa* and *E. coli* (15). As expected, in addition to the TonB1 and TonB3 single mutants being affected in uptake, the combination of double *tonB* mutants were more severely affected in the transport of enterobactin in both the experiments.

TonB2 sequence analysis

In order to determine whether the differential phenotype observed with *tonB2* mutants amongst the *C. jejuni* isolates was due to any differences in the protein at the amino acid level we sequenced the *tonB2* gene from *C. jejuni* 81-176. The multiple sequence alignment by Clustal W revealed that the amino acid sequences of *C. jejuni* NCTC 11168 TonB2 and *C. jejuni* 81-176 TonB2 are almost identical and/or conserved except at three positions (Figure 6). Interestingly, two out of these three positions involved substitution of threonine (polar) residue at positions 93 and 224 by isoleucine (hydrophobic

non-polar) residue. The third change identified was the substitution of isoleucine at position 184 by serine (polar) residue. These substitutions might have an effect on the three-dimensional structure of the protein. We also observed that arginine residue at position 199 was conserved in TonB2 and TonB3 of *C. jejuni* NCTC, and TonB2 of *C. jejuni* 81-176. This conservation of arginine residue might have implications on the interaction with the TonB dependent outer membrane receptors.

Animal Models

The *tonB* mutants are significantly affected in their ability to colonize the ceca of birds

In order to determine the potential benefits that *C. jejuni* wild-type strains have by possessing the multiple TonB systems, we assessed their role by non-competitive and competitive colonization of the chicken ceca. The chick model is an established and well characterized model for the assessment of the colonization potential of *C. jejuni* (11, 27, 29). *C. jejuni* colonizes the intestinal tract of chicks as a commensal (14).

Non-competitive cecal colonization

The colonization ability of the *tonB* mutants and the wild-type *C. jejuni* NCTC 11168 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 and the ceca were recovered. The cecal contents checked for the wild-type strain and the *tonB* mutants recovery titer as described in the materials and methods. At a 5% level of significance (Non-Parametric Mann-Whitney Rank Sum test), all the *tonB* mutants were

found to be significantly affected in colonization as compared to the wild-type strain except the *tonB2* mutant (p=0.095), which was found to be not affected in colonization (Figure 7). The single *tonB1* mutant and the single *tonB3* mutant as well as all the double *tonB* mutants were significantly reduced in their colonization of the ceca. These results indicate that *tonB1* and *tonB3* are essential for cecal colonization of *C. jejuni*. The level of colonization indicated the double mutants to be severely affected than the single mutants. The recovery of *tonB1tonB3* double mutant was below the detection limit of 100 cfu/gram ceca.

Competitive cecal colonization

The ability of the *tonB* mutants to colonize the chicken ceca was further tested in competition with the wild-type *C. jejuni* NCTC 11168 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. The experiment was performed as described in materials and methods section. The 1:1 ratio of the mixed inoculum was confirmed by the determination of cfu. Ninety-six hours post challenge, the ceca were recovered and the contents were then plated on selective media with and without kanamycin or chloramphenicol antibiotics. The bacterial titer of the wild-type and mutant strains was recorded and the competitive index for each bird was calculated as the ratio of the mutant to the wild-type recovered. The competitive indices of each mutant group were compared by Student *t* test at a 5% level of significance. As shown in Figure 8, with the exception of the single *tonB2* mutants, all the other single and double *tonB* mutants were significantly affected in their ability to

colonize the gastrointestinal tract of birds ($p<0.0001$). These results corroborated the findings of the non-competitive chick colonization assays. The double mutants were severely attenuated followed by the *tonB3* and *tonB1* single mutants.

This *in vivo* phenotype of *tonB* mutants reflected the iron limited environment of the chicken ceca. The growth characteristics of the *tonB* mutants *in vitro* was observed to be not significantly different ($p>0.05$) than the wild-type strain (Figure 2). Therefore, the inability of the mutants to grow *in vivo* is indicative of a significant *in vivo* specific defect.

Colostrum-deprived neonatal piglets colonization model

To determine whether the *tonB* mutants were defective in their ability to colonize the intestine of colostrum-deprived neonatal piglets, competitive colonization assays were performed. The use of colostrum-deprived neonatal piglets has been documented to be a relevant *in vivo* model for human Campylobacteriosis (2). Babakhani *et.al* reported the induction of diarrhea in experimentally infected piglets, which was characterized by the presence of mucus and blood mimicking the signs of human *Campylobacter* infection. Moreover, the gastrointestinal tract of piglets is very similar to the human gastrointestinal tract (27). Also, the neonatal piglet model has been established and used for the screening of other iron acquisition mutants of *C. jejuni* strains (Stintzi unpublished data).

C. jejuni NCTC 11168 wild-type strain and its *tonB* mutants were tested for their ability to colonize the intestinal tract of colostrum-deprived neonatal piglets by competitive colonization assays as described in the materials and methods section. Two piglets were tested for competitive colonization of each *tonB* mutant and the wild-type strain. Each

piglet was orally challenged with 1:1 mixed inocula containing the wild-type strain and each *tonB* mutant. The mixed inocula ratio was confirmed to be 1:1 by cfu determination. Seventy-two hours post challenge, the piglets were euthanized and the intestinal segments were collected. The contents of the intestinal segments were plated on selective media and the bacterial titers of the wild-type strain and the *tonB* mutants were determined. Then the competitive index was calculated for each piglet. The competitive colonization potential was determined for all the single and double *tonB* mutants and analyzed at a 5% level of significance by Student *t* test. The competitive index was tested for each group, for each piglet and was also tested organ-wise. The bacterial recovery was checked in the duodenum, jejunum, ileum, cecum and colon and the competitive index was determined. All the three single *tonB* mutants were significantly affected ($p<0.0001$) in their colonization potential as compared to the wild type strain (Figure 9 A, B, C). Since the wild type strain was recovered from all the intestinal segments, this data suggests that *C. jejuni* colonizes all the intestinal segments. As expected, the double *tonB* mutants were more severely affected in their colonization levels than the single *tonB* mutants ($p<0.00001$) (Figure 9 D, E, F). Our study demonstrated that the colonization potential of the single *tonB* mutants was significantly (though not severely) attenuated, which is likely due to the functional compensation by the other TonBs. The mutations in the two TonBs of the same *C. jejuni* strain further decreased the iron acquisition ability as compared to the single *tonB* mutants and therefore severely affected the colonization potential.

In contrast to the chick colonization data, the *tonB2* mutant was found to be significantly defective in the colonization of the piglet model. This differential phenotype in the

colonization potentials of the *tonB2* mutant in the two animal models studied highlights the importance of assessing the mutants in multiple relevant animal models for thorough characterization of the function of a gene or protein. The differential phenotype exhibited by the *tonB2* mutant suggests the expression of the TonB systems to be likely a host specific effect. Furthermore, different TonBs might play a different role in the colonization of the gastrointestinal tract of the host.

Our *in vivo* results are similar to the *in vivo* phenotypes observed with the characterization of TonB transport systems in several other Gram-negative bacteria. For instance, in *V. cholerae* each of the *tonB* mutants had a moderate reduction in the competitive colonization with the wild-type strain, indicative of a role of each TonB system *in vivo* (25, 38). In *Salmonella enterica serovar typhimurium,* its *tonB* mutant was significantly attenuated in the infection of mice by intragastric route, but not via intraperitoneal route of infection (41). *Pseudomonas aeruginosa tonB* mutant was defective in experimental infection of immunocompromised mice (40). In *Haemophilus influenzae* type b, the *tonB* mutant was defective in infection of rats by intraperitoneal and intanasal route of infection (18). These reports indicate that the role of TonB dependent bacterial infection varies depending on the site of infection. Recently, it has been shown in *V. anguillarium* that only one of the TonBs is essential for virulence (39). Our data demonstrated the additive and functionally redundant roles of *C. jejuni* TonBs in iron acquisition within the gastrointestinal tract of the host. Nevertheless, complete loss of TonB function cannot be confirmed due to lack of a triple *tonB* mutant in this study.

Concluding remarks

From this study we can conclude that the TonB proteins are essential for the utilization of siderophores, heme compounds and other iron sources by *C. jejuni* and that the transport of the compounds across the outer membrane is proton dependent. The multiple TonBs in *C. jejuni* served specific and redundant functions in the transport of various iron sources. The outcome of growth promotion assays and uptake experiments suggests that all *C. jejuni* isolates exhibit a characteristic pattern in the utilization of iron from different sources. This is likely related to the presence or absence of functional multiple TonB system(s). The *in vivo* colonization experiments with the commensal chicken model and pathogenicity testing neonatal piglet model clearly demonstrated the significant contribution of TonBs in the colonization of the gastrointestinal tract by *C. jejuni*. Reduced colonization of the *tonB* mutants due to the inactivation of *tonB* genes might also affect other mechanisms besides iron acquisition pertaining to growth and infectivity of *C. jejuni in vivo*. It is evident in *E. coli* that TonB functions for Vitamin B_{12} uptake in addition to ferric iron acquisition (4). Exploring the possible functions of TonBs in *C. jejuni* might further contribute in better understanding of the role of TonBs in colonization and pathogenesis.

References

1. Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones. 2003. Bacterial iron homeostasis. FEMS Microbiol Rev 27:215-37.
2. Babakhani, F. K., G. A. Bradley, and L. A. Joens. 1993. Newborn piglet model for Campylobacteriosis. Infect Immun 61:3466-75.
3. Baig, B. H., I. K. Wachsmuth, and G. K. Morris. 1986. Utilization of exogenous siderophores by *Campylobacter* species. J Clin Microbiol 23:431-3.
4. Bassford, P. J., Jr., C. Bradbeer, R. J. Kadner, and C. A. Schnaitman. 1976. Transport of vitamin B12 in *tonB* mutants of *Escherichia coli*. J Bacteriol 128:242-7.
5. Braun, V. 1995. Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol Rev 16:295-307.
6. Braun, V. 2003. Iron uptake by *Escherichia coli*. Front Biosci 8:s1409-21.
7. Day, W. A., Jr., J. L. Sajecki, T. M. Pitts, and L. A. Joens. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. Infect Immun 68:6337-45.
8. Demir, M., and I. Kaleli. 2004. Production by *Escherichia coli* isolates of siderophore and other virulence factors and their pathogenic role in a cutaneous infection model. Clin Microbiol Infect 10:1011-4.
9. Dorrell, N., J. A. Mangan, K. G. Laing, J. Hinds, D. Linton, H. Al-Ghusein, B. G. Barrell, J. Parkhill, N. G. Stoker, A. V. Karlyshev, P. D. Butcher, and B. W. Wren. 2001. Whole genome comparison of *Campylobacter jejuni* human isolates

using a low-cost microarray reveals extensive genetic diversity. Genome Res 11:1706-15.

10. Field, L. H., V. L. Headley, S. M. Payne, and L. J. Berry. 1986. Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. Infect Immun 54:126-32.

11. Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell. 2004. The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. J Bacteriol 186:503-17.

12. Gorbacheva, V. Y., G. Faundez, H. P. Godfrey, and F. C. Cabello. 2001. Restricted growth of *ent*(-) and *tonB* mutants of *Salmonella enterica serovar typhi* in human Mono Mac 6 monocytic cells. FEMS Microbiol Lett 196:7-11.

13. Guerry, P., J. Perez-Casal, R. Yao, A. McVeigh, and T. J. Trust. 1997. A genetic locus involved in iron utilization unique to some *Campylobacter* strains. J Bacteriol 179:3997-4002.

14. Hendrixson, D. R., and V. J. DiRita. 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol 52:471-84.

15. Hoegy, F., H. Celia, G. L. Mislin, M. Vincent, J. Gallay, and I. J. Schalk. 2005. Binding of iron-free siderophore a common feature of siderophore outer membrane transporters of *Escherichia coli* and *Pseudomonas aeruginosa*. J Biol Chem.

16. Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells. 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology 151:243-57.

17. Jarosik, G. P., and E. J. Hansen. 1994. Identification of a new locus involved in expression of *Haemophilus influenzae* type b lipooligosaccharide. Infect Immun 62:4861-7.

18. Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen. 1994. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type b. Infect Immun 62:2470-7.

19. Ketley, J. M. 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology 143 (Pt 1):5-21.

20. Knosp, O., M. von Tigerstrom, and W. J. Page. 1984. Siderophore-mediated uptake of iron in *Azotobacter vinelandii*. J Bacteriol 159:341-7.

21. Larsen, R. A., P. S. Myers, J. T. Skare, C. L. Seachord, R. P. Darveau, and K. Postle. 1996. Identification of TonB homologs in the family Enterobacteriaceae and evidence for conservation of TonB-dependent energy transduction complexes. J Bacteriol 178:1363-73.

22. Leonard, E. E., 2nd, T. Takata, M. J. Blaser, S. Falkow, L. S. Tompkins, and E. C. Gaynor. 2003. Use of an open-reading frame-specific *Campylobacter jejuni* DNA microarray as a new genotyping tool for studying epidemiologically related isolates. J Infect Dis 187:691-4.

23. Leung, K. Y., and B. B. Finlay. 1991. Intracellular replication is essential for the virulence of *Salmonella typhimurium*. Proc Natl Acad Sci U S A 88:11470-4.

24. Mey, A. R., and S. M. Payne. 2003. Analysis of residues determining specificity of *Vibrio cholerae* TonB1 for its receptors. J Bacteriol 185:1195-207.

25. Mey, A. R., and S. M. Payne. 2001. Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. Mol Microbiol 42:835-49.

26. Moeck, G. S., and J. W. Coulton. 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol Microbiol 28:675-81.

27. Newell, D. G. 2001. Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models. Symp Ser Soc Appl Microbiol:57S-67S.

28. Otto, B. R., A. M. Verweij-van Vught, and D. M. MacLaren. 1992. Transferrins and heme-compounds as iron sources for pathogenic bacteria. Crit Rev Microbiol 18:217-33.

29. Palyada, K., D. Threadgill, and A. Stintzi. 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol 186:4714-29.

30. Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-8.

31. Pesci, E. C., D. L. Cottle, and C. L. Pickett. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect Immun 62:2687-94.

32. Pickett, C. L., T. Auffenberg, E. C. Pesci, V. L. Sheen, and S. S. Jusuf. 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. Infect Immun 60:3872-7.

33. Poly, F., D. Threadgill, and A. Stintzi. 2004. Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. J Bacteriol 186:4781-95.

34. Postle, K. 1993. TonB protein and energy transduction between membranes. J Bioenerg Biomembr 25:591-601.

35. Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol 54:881-941.

36. Reeves, S. A., A. G. Torres, and S. M. Payne. 2000. TonB is required for intracellular growth and virulence of *Shigella dysenteriae*. Infect Immun 68:6329-36.

37. Richardson, P. T., and S. F. Park. 1995. Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system. Microbiology 141 (Pt 12):3181-91.

38. Seliger, S. S., A. R. Mey, A. M. Valle, and S. M. Payne. 2001. The two TonB systems of *Vibrio cholerae*: redundant and specific functions. Mol Microbiol 39:801-12.

39. Stork, M., M. Di Lorenzo, S. Mourino, C. R. Osorio, M. L. Lemos, and J. H. Crosa. 2004. Two tonB systems function in iron transport in *Vibrio anguillarum*, but only one is essential for virulence. Infect Immun 72:7326-9.

40. Takase, H., H. Nitanai, K. Hoshino, and T. Otani. 2000. Requirement of the *Pseudomonas aeruginosa tonB* gene for high-affinity iron acquisition and infection. Infect Immun 68:4498-504.

41. Tsolis, R. M., A. J. Baumler, F. Heffron, and I. Stojiljkovic. 1996. Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. Infect Immun 64:4549-56.

42. van Vliet, A. H., and J. M. Ketley. 2001. Pathogenesis of enteric *Campylobacter* infection. Symp Ser Soc Appl Microbiol:45S-56S.

43. van Vliet, A. H., J. M. Ketley, S. F. Park, and C. W. Penn. 2002. The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. FEMS Microbiol Rev 26:173-86.

44. Wandersman, C., and P. Delepelaire. 2004. Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol 58:611-47.

45. Wooldridge, K. G., and P. H. Williams. 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiol Rev 12:325-48.

Table 1: Bacterial plasmids used in this study

Strain	Relevant characteristic(s) [a]	Source or reference
pUC19	cloning and suicidal vector,	Biolabs
pAS16	pUC19 carrying *tonB1*	This study
pAS20	pUC19 carrying Δ*tonB1*	This study
pAS23	pUC19 carrying Δ*tonB1:: Km*^{r}	This study
pAS17	pUC19 carrying *tonB2*	This study
pAS24	pUC19 carrying Δ*tonB2*	This study
pAS176	pUC19 carrying Δ*tonB2::Cm*^{r}	This study
pAS41	pUC19 carrying *tonB3*	This study
pAS42	pUC19 carrying Δ*tonB3*	This study
pAS356	pUC19 carrying Δ*tonB3::Km*^{r}	This study
pAS348	pUC19 carrying Δ*tonB1:Cm*^{r}	This study
pRY111	*Cm*^{r} resistance gene	Guerry
pILL600	*Km*^{r} resistance gene	Labigne

a Amp^r , ampicillin resistant

Cm^r , chloramphenicol resistance gene

Km^r , kanamycin resistance gene

Table 2: Bacterial strains used in this study

Strain	Relevant characteristic(s)	Source or reference
E. coli DH5α	*endA1 hsdR17($r_K^-m_K^-$)supE44*	Invitrogen
C. jejuni strains		
AS144	*C. jejuni* NCTC 11168	NCTC
AS27	AS144Δ*tonB1*	This study
AS240	AS144Δ*tonB2*	This study
AS239	AS144Δ*tonB3*	This study
AS241	AS144Δ*tonB1*Δ*tonB2*	This study
AS242	AS144Δ*tonB2*Δ*tonB3*	This study
AS355	AS144Δ*tonB1*Δ*tonB3*	This study
AS53	*C. jejuni* ATCC 43431 (TGH	ATCC
AS183	AS53Δ*tonB2*	This study
AS235	AS53Δ*tonB3*	This study
AS356	AS53Δ*tonB2*Δ*tonB3*	This study
AS275	*C. jejuni* 81-176	Pickett
AS252	AS275Δ*tonB2*	This study

Table 3: Primers used in this study

Primer	DNA sequence from 5' to 3' (restriction site) [a]
TonB11	GGAAGATCTGCACCATTTAGATGAGCTTGC (*Bgl*II)
TonB12	GGAAGATCTCACTTGCTGAATAATTTGGCT (*Bgl*II)
TonB13	CGCGGATCCCAATATCAAACTTAACACAAACC
TonB14	CGCGGATCCGTTATGCCTATTGATTACAAT (*Bam*HI)
TonB21	GGAAGATCTCGGGCAATATAGATGTCAAATC
TonB22	GGAAGATCTTCTGATGATGCAAATATAGAAG
TonB23	CGCGCATCCTATATAAGAAGCTTGGTATTTATG
TonB24	CGCGGATCCCCACACTATAATGGAGATTTAG
TonB31	GGAAGATCTTGACTTGCTAGCAGTTGAAGAT
TonB32	GGCAGATCTCATTGATAGTAGCAGGAGCTTC
TonB33	GCCGGATCCACTATACGCATAGCTTCTAAA
TonB34	CGCGGATCCTCCACATTTTTGTTTATTTCACTA

[a] The restriction sites used for cloning are underlined and correspondingly shown in parenthesis

Table 4: Binding and invasion assays with INT-407 cells

	% Binding[a]	% Invasion[b]
C. jejuni NCTC 11168		
Wild type	0.0067 ± 0.00083	0.00032 ± 0.000078
Δ*tonB1*	0.0043 ± 0.00062	0.00036 ± 0.00006
Δ*tonB2*	0.0053 ± 0.00082	0.00041 ± 0.000099
Δ*tonB3*	0.0048 ± 0.00085	0.00063 ± 0.00005
Δ*tonB1B2*	0.0057 ± 0.00092	0.0004 ± 0.000062
Δ*tonB2B3*	0.0059 ± 0.00075	0.00043 ± 0.000044
Δ*tonB1B3*	0.00553 ± 0.00064	0.0003 ± 0.000062
C. jejuni TGH 9011		
Wild type	1.37 ± 0.087	0.37 ± 0.039
Δ*tonB2*	1.49 ± 0.11	0.36 ± 0.051
Δ*tonB3*	1.74 ± 0.61	0.63 ± 0.041
Δ*tonB2B3*	1.57 ± 0.075	0.57 ± 0.062
C. jejuni 81-176		
Wild type	1.42 ± 0.061	0.61 ± 0.11
Δ*tonB2*	1.42 ± 0.1	0.47 ± 0.05

a obtained by subtracting the number of invaded bacteria from the total number of bacteria recovered without gentamicin treatment

b percentage of inoculum recovered after gentamicin treatment

Table 5: Bioassay for *C. jejuni* NCTC 11168 (zone of growth measured as diameter in mm)

	$FeSO_4$	Enterobactin	Hemin	Hemoglobin
Wild type	14.75 ± 1.31	26 ± 1.91	25.25 ± 1.18	16 ± 1.68
Δ*tonB1*	15.25 ± 1.44	25.25 ± 1.65	21.75 ± 1.93	17.25 ± 1.80
Δ*tonB2*	15.25 ± 1.25	26 ± 1.35	21 ± 1	15.75 ± 1.75
Δ*tonB3*	15.25 ± 1.25	0	22.75 ± 1.11	16.25 ± 1.25
Δ*tonB1B2*	16 ± 1.63	24.25 ± 1.65	19.25 ± 1.11	13.5 ± 0.96
Δ*tonB2B3*	12.25 ± 0.63	0	0	0
Δ*tonB1B3*	16 ± 1.35	0	23 ± 1.47	16.25 ± 1.65

Table 6: *C. jejuni* bioassay results

	$FeSO_4$	Enterobactin	Hemin	Hemoglobin	Lysine CAM
C. jejuni NCTC 11168					
Wild type	+	+	+	+	+
Δ*tonB1*	+	+	+	+	+
Δ*tonB2*	+	+	+	+	+
Δ*tonB3*	+	_	+	+	_
Δ*tonB1B2*	+	+	+	+	+
Δ*tonB2B3*	+	_	_	_	_
Δ*tonB1B3*	+	_	+	+	_
C. jejuni TGH 9011					
Wild type	+	_	+	+	_
Δ*tonB2*	+	_	+	+	_
Δ*tonB3*	+	_	_	_	_
Δ*tonB2B3*	+	_	_	_	_
C. jejuni 81-176					
Wild type	+	_	+	+	+
Δ*tonB2*	+	_	_	_	_

+, positive, forming a zone of growth around the iron source

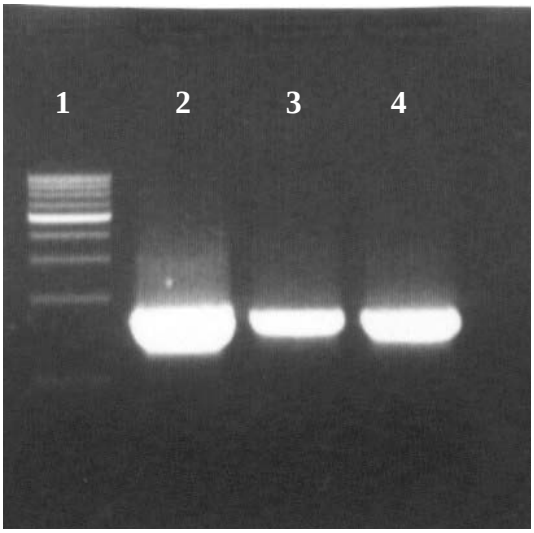
-, negative, no zone of growth around the iron source

Figure 1: Gel pictures of the wild type *C. jejuni* NCTC 11168 genes and the double *tonB* mutant. **A)** 0.9% agarose gel picture of PCR product obtained with the wild type *C. jejuni* NCTC 11168. *tonB1* (lane 2), *tonB2* (lane 3), *tonB3* (lane 4) and standard 1 Kb DNA ladder (lane 1). The approximate sizes of *tonB1*, *tonB2* and *tonB3* PCR products are 1623 bp, 1668 bp and 1600 bp respectively.

B) 0.9% agarose gel picture of the PCR products obtained with the double *tonB2tonB3* mutants of *C. jejuni* NCTC 11168. *tonB1* (lane 2), Δ*tonB2* (lane 3), Δ*tonB3* (lane 4) and the standard 1 Kb DNA ladder (lane 1).

Figure 1:

A)



B)

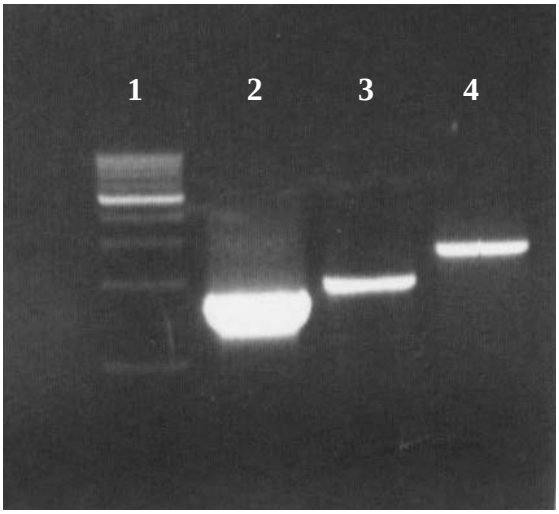
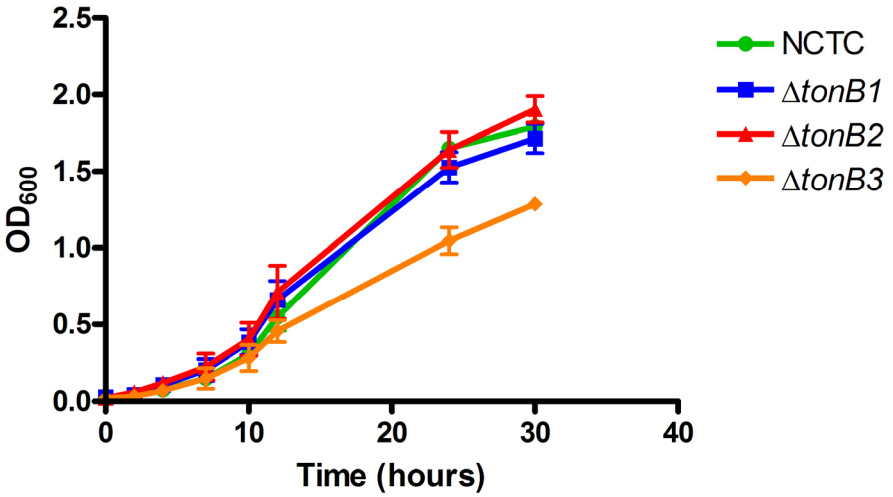


Figure 2: Comparative growth of *C. jejuni* NCTC 11168 wild type and *tonB* mutants in MH biphasic medium. The data represents the mean of three independent experiments ± standard error. **A)** Single *tonB* mutants. **B)** Double *tonB* mutants.

Figure 2:

A)


2.5
2.0
1.5
1.0
0.5
0.0
OD600
0
10
20
30
40
Time (hours)
NCTC
ΔtonB1
ΔtonB2
ΔtonB3

B)

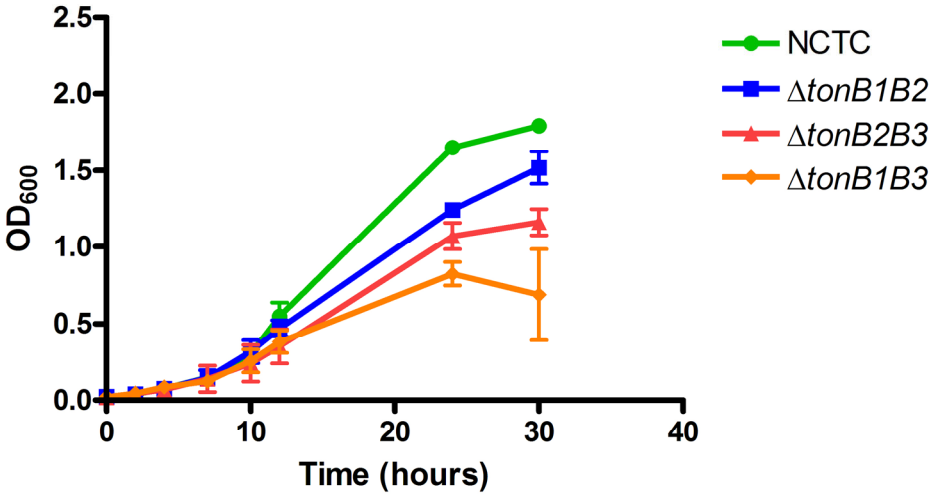
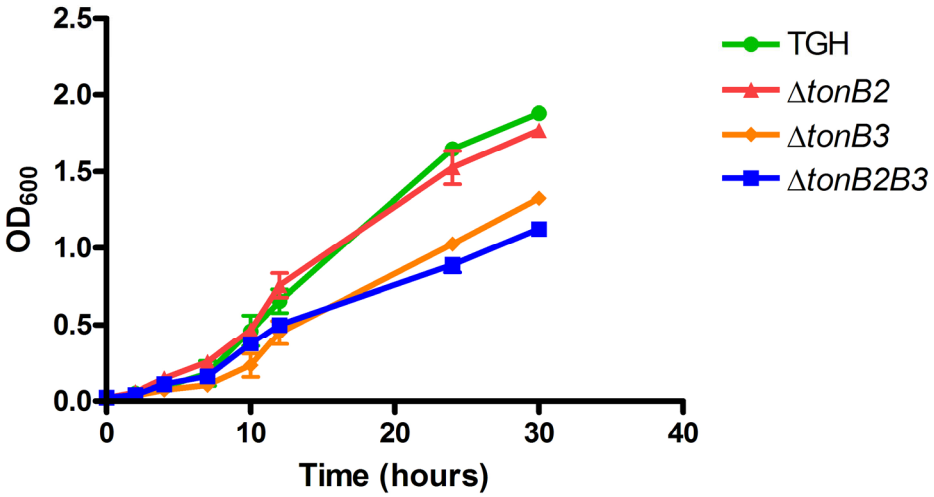

2.5
2.0
1.5
1.0
0.5
0.0
OD600
0
10
20
30
40
Time (hours)
NCTC
ΔtonB1B2
ΔtonB2B3
ΔtonB1B3

Figure 3: Comparative growth of *C. jejuni* TGH 9011 and *C. jejuni* 81-176 wild type and *tonB* mutants in MH biphasic medium. The data represents the mean of three independent experiments ± standard error. **A)** *C. jejuni* TGH growth curves. **B)** *C. jejuni* 81-176 growth curves.

Figure 3:

A)


2.5
2.0
1.5
1.0
0.5
0.0
OD600
0
10
20
30
40
Time (hours)
TGH
ΔtonB2
ΔtonB3
ΔtonB2B3

B)

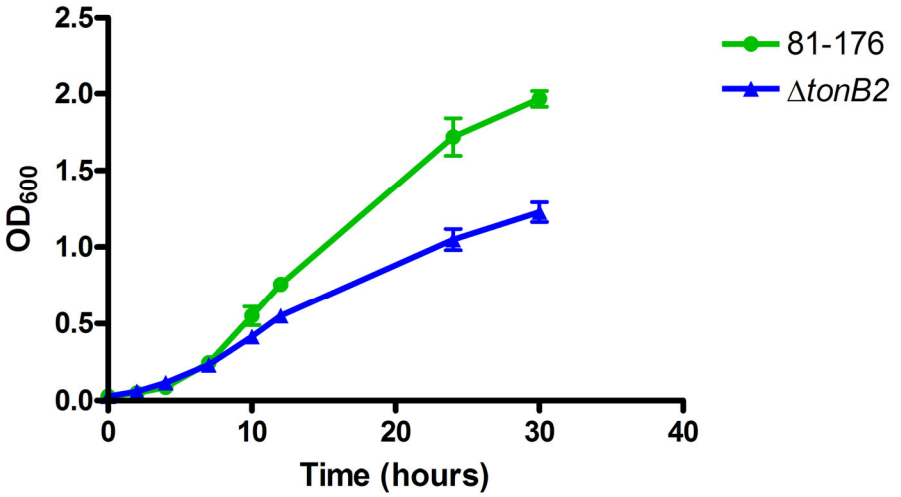
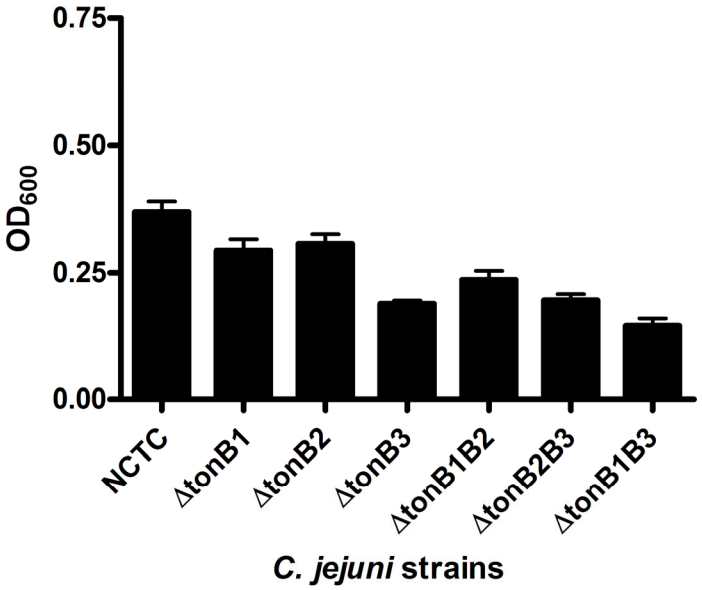

2.5
2.0
1.5
1.0
0.5
0.0
OD600
0
10
20
30
40
Time (hours)
81-176
ΔtonB2

Figure 4: Comparative growth of *C. jejuni* NCTC 11168 wild type and *tonB* mutants in MEMα medium. *C. jejuni* strains were grown overnight in MH biphasic medium, harvested, washed and resuspended in MEMα before inoculating into fresh iron restricted MEMα (A) or MEMα supplemented with 40 μM $FeSO_4$ (B). The data represents the mean OD600 per ml measured at 24 hours (early stationary phase) from three independent experiments ± standard error.

Figure 4:

A)


0.75
0.50
0.25
0.00
OD600
NCTC
ΔtonB1
ΔtonB2
ΔtonB3
ΔtonB1B2
ΔtonB2B3
ΔtonB1B3
C. jejuni strains

B)

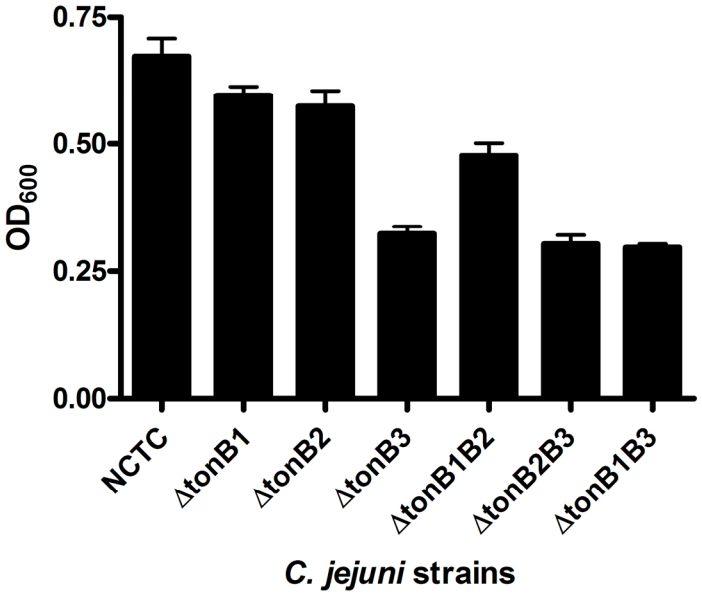
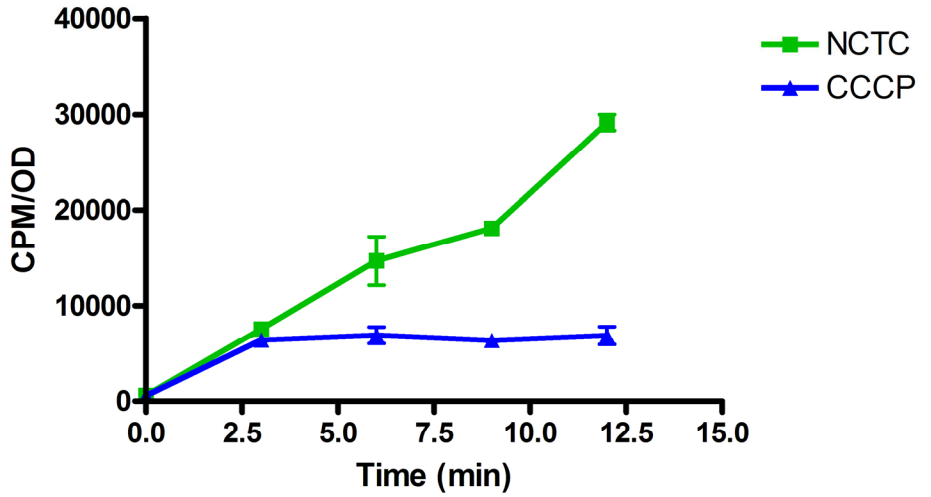

0.75
0.50
0.25
0.00
OD600
NCTC
ΔtonB1
ΔtonB2
ΔtonB3
ΔtonB1B2
ΔtonB2B3
ΔtonB1B3
C. jejuni strains

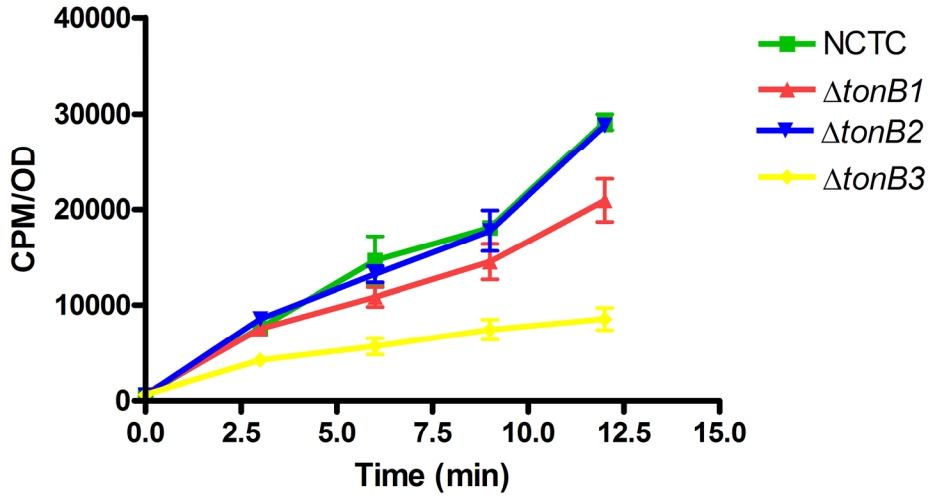
Figure 5: Ferric-enterobactin uptake assay. *C. jejuni* NCTC 11168 strain was grown over night in iron limited MEMα medium, and harvested at mid-log phase for ferric-enterobactin (final concentration 0.018 μM) uptake experiment. Uptake was monitored for 12 minutes following the addition of the $^{55}Fe^{3+}$-enterobactin complex.
A) Enterobactin mediated iron transport in *C. jejuni* NCTC 11168 strain in presence or absence of the proton ionophore, CCCP at 33μM. **B)** Comparative uptake of the single *tonB* mutants and the wild type *C. jejuni* NCTC 11168 strain. **C)** Comparative uptake of the double *tonB* mutants and the wild type *C. jejuni* NCTC 11168 strain.

Figure 5:

A)


40000
30000
20000
10000
0
CPM/OD
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time (min)
NCTC
CCCP

B)


40000
30000
20000
10000
0
CPM/OD
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time (min)
NCTC
ΔtonB1
ΔtonB2
ΔtonB3

C)

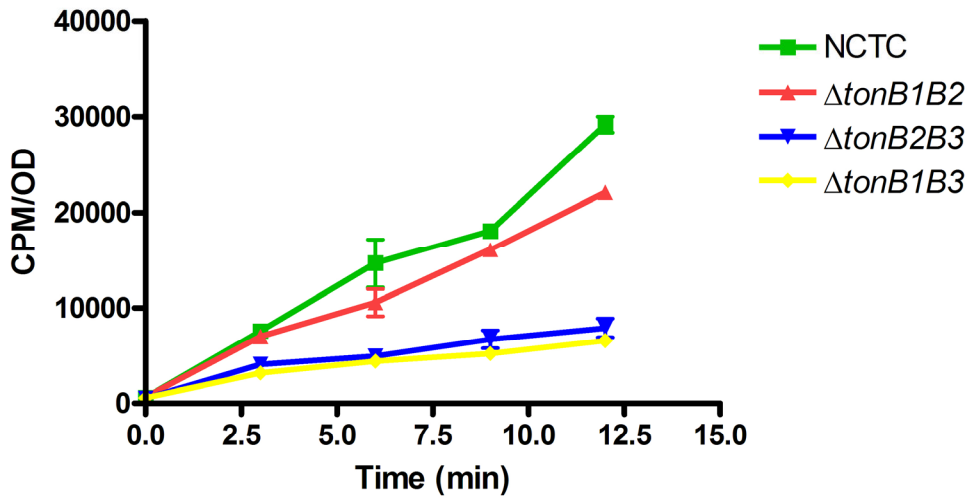

40000
30000
20000
10000
0
CPM/OD
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time (min)
NCTC
ΔtonB1B2
ΔtonB2B3
ΔtonB1B3

Figure 6: Clustal W Alignment of TonB sequences of *C. jejuni*. Multiple sequence alignment of TonB1, TonB2 and TonB3 amino acid sequences from *C. jejuni* NCTC 11168 and TonB2 from *C. jejuni* 81-176. Single, fully conserved residues are indicated by an asterisk, conservation of strong groups are indicated by a colon, conservation of weak groups are indicated by a dot and no markings below the alignment indicate non conserved residues. The numbers flanking the sequences refer to the last amino acids in the sequence. The arginine residue at position 199 is highlighted in red color and is underlined.

Figure 6:

```
81176           MKTLFLNHKYQASYITFIVFIPLLFVIFHSNDFFKMEIK---NEDSFSLAIKQFTQTNP- 56
tonB2NCTC       MKTLFLNHKYQASYITFIVFIPLLFVIFHSNDFFKMEIK---NEDSFSLAIKQFTQTNP- 56
tonB3NCTC       MKTFISNHKNQSSFITLFVFTPLFFVFLYSKDFLHIQPNETIKENKFNMAIKHFVQNSSD 60
tonB1NCTC       MKSSVIFGFVLSLILHTLVLMFFLFSFYTQEKSSGVDFKQGAEFTSIMMVSEFPIGELKE 60
                **: .      :  :  :*:  ::* :  .:.   :: :   :  .: :. :

81176           TNETKPTESIIEPIKPKPQPKVIKKTPEKIQKKVKKIPPHPIPN--KTPIAPTQEVKTFA 114
tonB2NCTC       TNETKPTESIIEPIKPKPQPKVIKKTPEKMQKKIKKTPPHPIPN--KTPIAPTQEVKTFA 114
tonB3NCTC       MKPTQPTQTIQEPSNVQP------KEPVQEIKKIKPRKEKPIAK--PKKIIPPANAKAIS 112
tonB1NCTC       VSIDQKKSNSQDKNKKQDERISFNSQDKNAVLKVQKKIEKQDENQAQKEIANASENSKFK 120
                 .  : ...  :  : :       .   :   *::    :   :   . *  . : . :

81176           KTT-DTNVKPKITQLTQGK------DNHPVLKEIQKAIQQAQFYPRQAKKMRMQGTVKVE 167
tonB2NCTC       KTT-DTNVKPKITQLTQGK------DNHPVLKEIQKAIQQAQFYPRQAKKMRMQGTVKVE 167
tonB3NCTC       QPKKDTNMQQQTPQASSYQS-VSLTSNSELLKEIKSAIDEALIYPRQARKMRMSGEVLVE 171
tonB1NCTC       NESLSAPLQSNKDKTQTIVSGNAKEQVKSYQALLMAHLTKFKKYPQEAIMQKQEGVVRIR 180
                : . .: :: :  :           .       :   : :   **::*   : .* * :.

81176           FLWKENKTLADLKIIESSGYDLLDKSALESIRKAS------LNFPHYNGDLRITLPIIYD 221
tonB2NCTC       FLWKENKTLADLKIIEISGYDLLDKSALESIRKAS------LNFPHYNGDLRITLPIIYD 221
tonB3NCTC       FTWTKEKKLENLKILKPSKYDFLNKSALETIRIAS------KKFPQYEKTFHIKIPLVYK 225
tonB1NCTC       VSIDESGNVLSKELKKSCPYAALNDEALSLFKRASPLPKPPKEMLKNGDKISFVMPIDYN 240
                .   :. .: . :: : . *  *:..**. :: **       :: :    : : :*: *.

81176           FKILR--- 226
tonB2NCTC       FKTLRG-- 227
tonB3NCTC       LS------ 227
tonB1NCTC       IKDYLGKK 248
                :.
```

Figure 7: Non competitive chick colonization assay with *tonB* mutants of *C. jejuni* NCTC 11168. Comparative colonization properties were tested in the wild-type strain and the *tonB* mutants of *C. jejuni* NCTC 11168. Five birds per group were orally challenged with 10^4 cfu. The minimum detection limit of this assay was 100 cfu per gram of ceca. The columns represent the means and the error bars indicate the standard errors. Non parametric Mann-Whitney Rank Sum test was used for statistical analysis. * indicates statistically significant difference at $p<0.0001$ for the single mutants and $p<0.00001$ for the double mutants.

Figure 7:

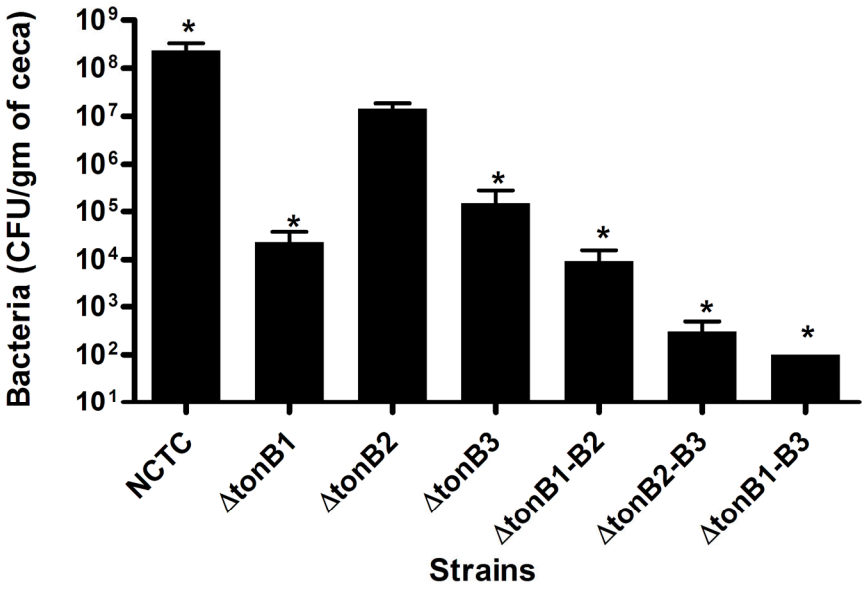

Bacteria (CFU/gm of ceca)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
*
NCTC
ΔtonB1
ΔtonB2
ΔtonB3
ΔtonB1-B2
ΔtonB2-B3
ΔtonB1-B3
Strains

Figure 8: Competitive chicken colonization studies with the *tonB* mutants of *C. jejuni* NCTC 11168. A 1:1 mixed inocula of the wild-type strain and Δ*tonB* strains were orally inoculated into five birds. The *in vivo* competitive index is the ratio of the output mutant to the wild-type strain recovered divided by the ratio of the input mutant to the wild-type strain inoculated. The minimum detection limit of this assay was 100 cfu per gram of ceca. The data represents the mean ± standard errors. * indicates a statistical significance of $p<0.0001$.

Figure 8:

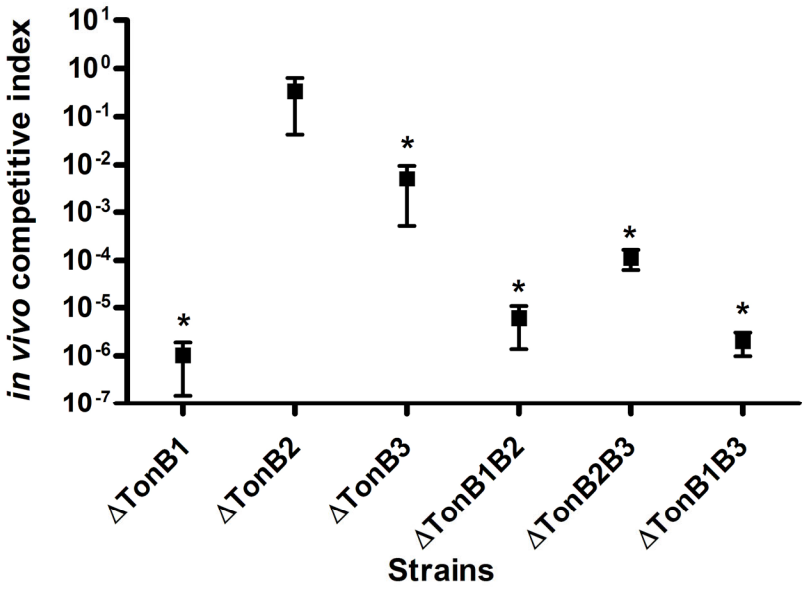
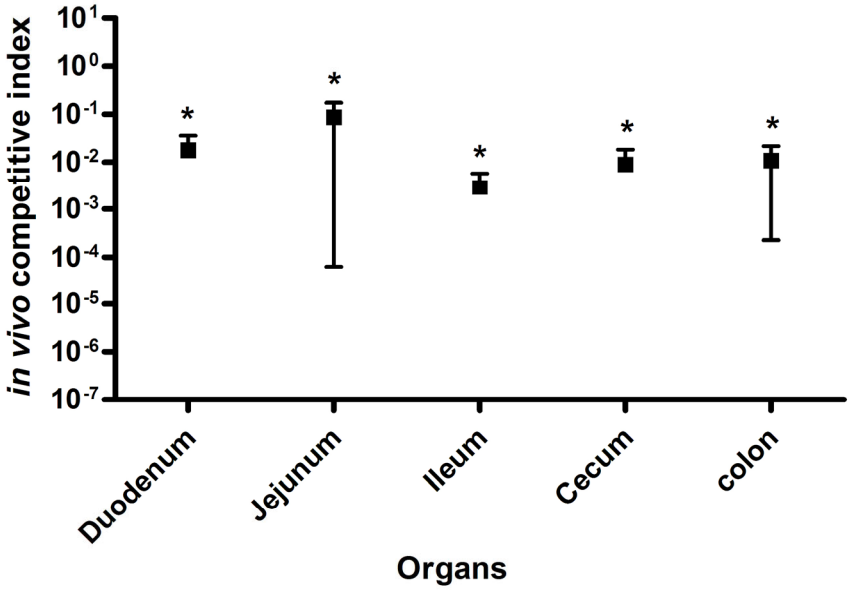

in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
*
*
*
*
ΔTonB1
ΔTonB2
ΔTonB3
ΔTonB1B2
ΔTonB2B3
ΔTonB1B3
Strains

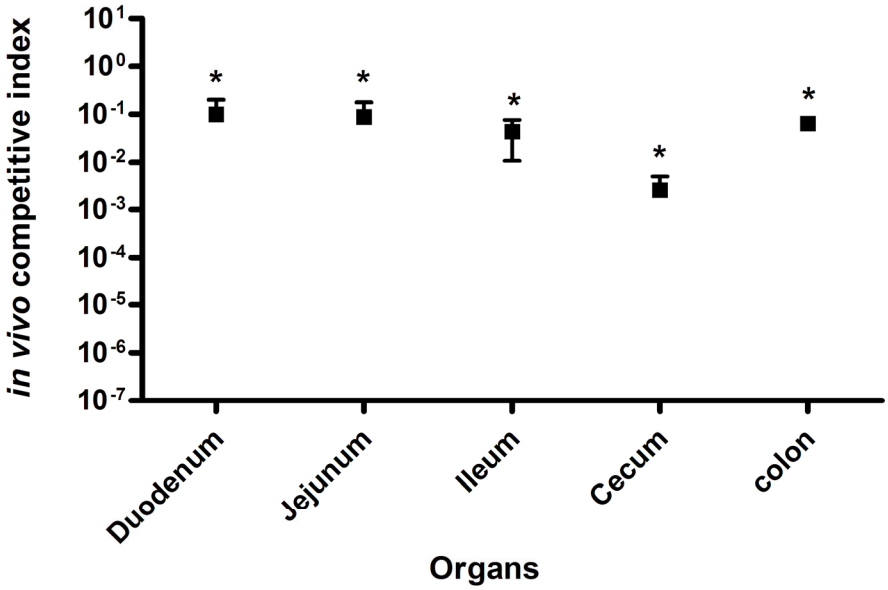
Figure 9: Colostrum-deprived neonatal piglet competitive colonization assay. The competitive colonization ability of the wild-type *C. jejuni* NCTC 11168 strains and its *tonB* mutants were assessed. Two piglets were orally challenged with a 1:1 mixed inocula of the wild-type strain and the tonB mutant. 72 hours post inoculation, the pigs were euthanized and the intestinal segments, duodenum, jejunum, ileum, cecum and colon were collected. The intestinal contents were checked for the wild-type strain and the *tonB* mutant titers. The CI was calculated as the ratio of the output mutant to the wild-type strain recovered divided by the ratio of the input mutant to the wild-type strain inoculated. The minimum detection limit of this assay was 100 cfu per gram of intestine. The data represents the mean ± the range of the values. This figure shows the CI for the mutants *tonB1* (A), Δ*tonB2* (B), Δ*tonB3* (C), Δ*tonB1B2* (D), Δ*tonB2B3* (E) and Δ*tonB1B3* (F). * indicates a statistical significance with a $p<0.0001$.

Figure 9:

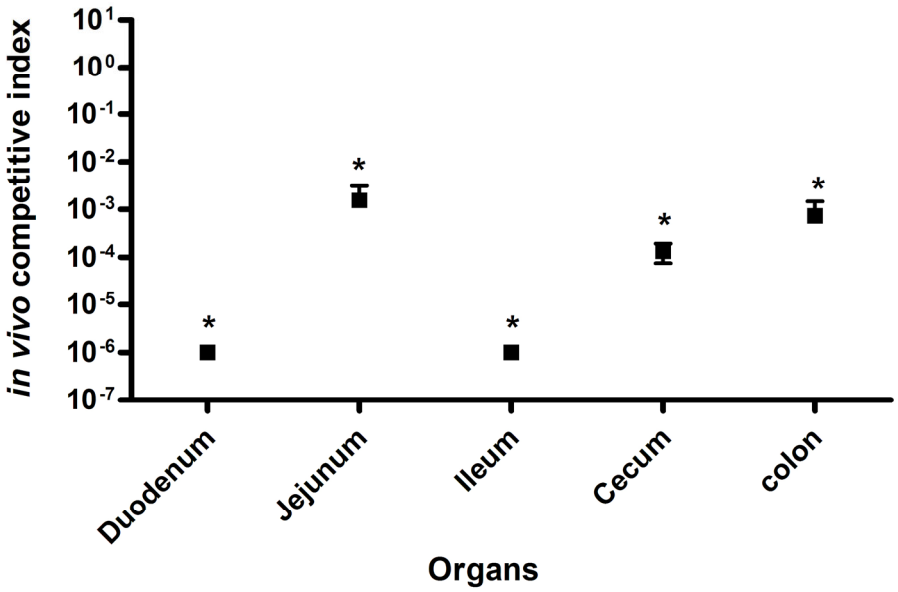
A)


in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

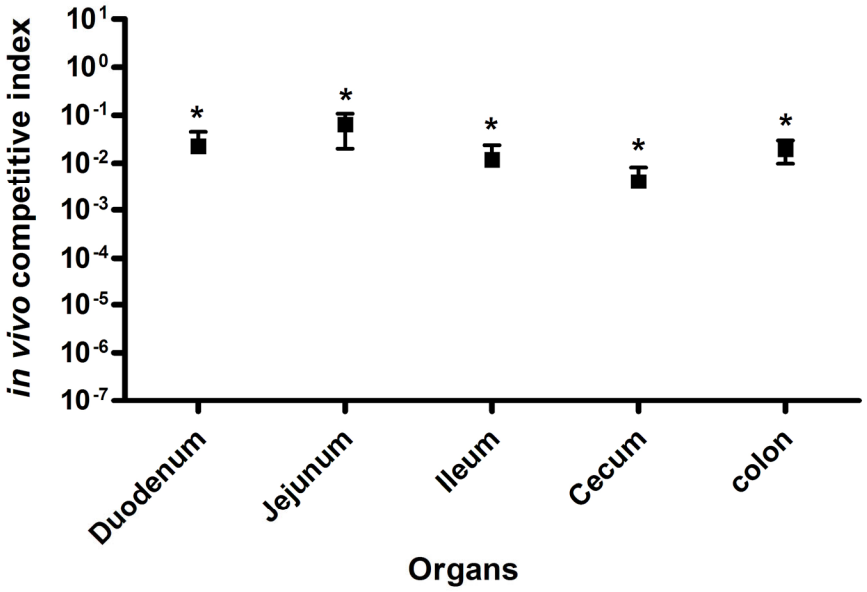
B)


in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

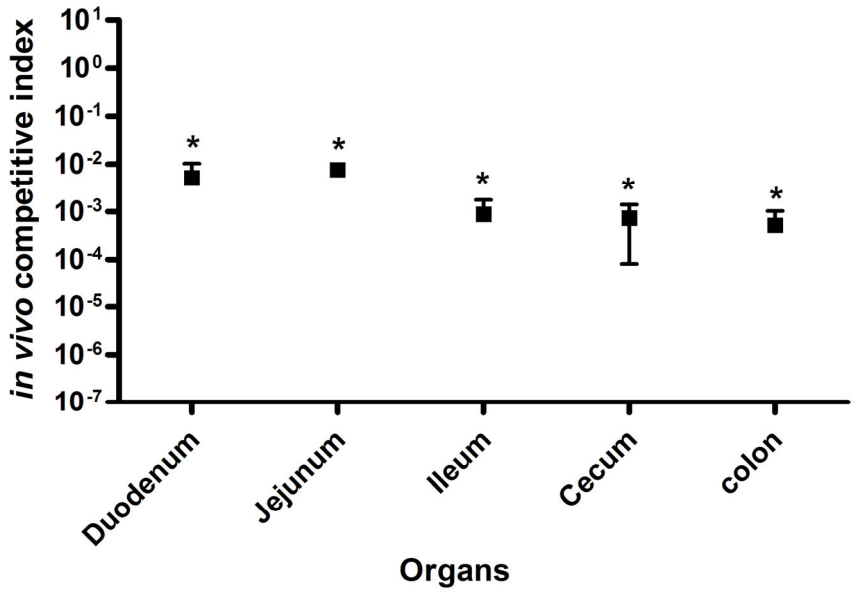
C)


in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

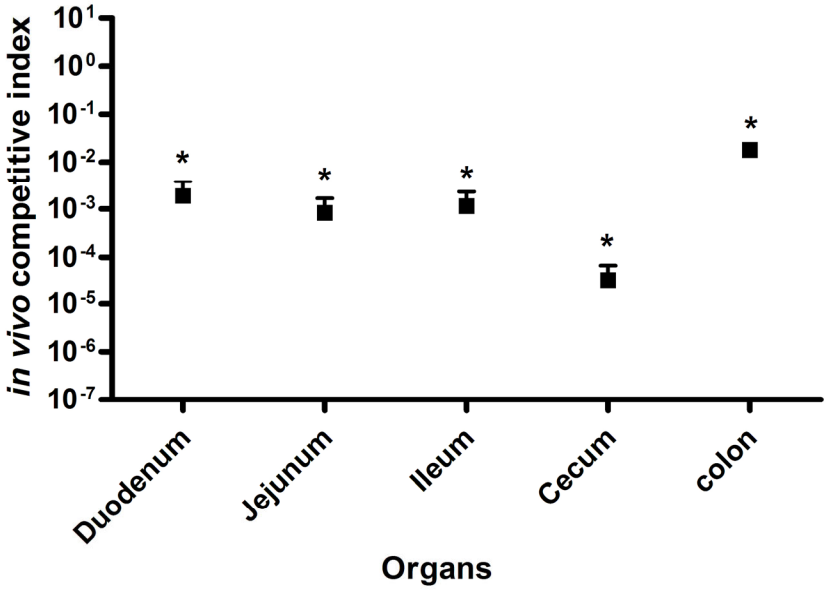
D)


in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

E)


in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

F)


in vivo competitive index
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
*
Duodenum
Jejunum
Ileum
Cecum
colon
Organs

Chapter V

Conclusion

The global aim of this project was to decipher the molecular mechanisms of iron acquisition in the food-borne pathogen, *C. jejuni*, since acquisition of iron has been demonstrated to be a key factor in the establishment of many bacterial infections. The complete genome sequence of *C. jejuni* NCTC 11168 suggested the presence of a putative ferrous iron transporter protein, FeoB and the presence of three putative TonB ExbB ExbD energy transduction complexes. We hypothesized that FeoB dependent ferrous iron transport and TonB mediated ferric iron transport systems were functional in *C. jejuni* and were important colonization determinants of *C. jejuni* infection. To test these hypotheses, we adopted a similar inactivation strategy by constructing knock-out mutants in *feoB* and *tonB* genes, and characterizing them *in vitro* and *in vivo*. We assessed the knock-out phenotypes for the poorly invasive and highly invasive *C. jejuni* isolates.

For the first objective, to characterize the FeoB system in *C. jejuni*, the *feoB* gene was knocked out by insertional inactivation using antibiotic resistance cassette. A battery of *in vitro* experiments was conducted to characterize the *feoB* mutants *in vitro*. By *in vitro* growth assays, we demonstrated no difference in the growth pattern of the *feoB* mutants and the wild-type strain. Using $^{55}Fe^{2+}$ uptake assays, we showed that ferrous iron uptake

was a proton dependent active process. The *feoB* mutant was significantly affected in iron uptake and was demonstrated to have decreased cellular iron accumulation. The role of FeoB in the intracellular survival of *C. jejuni* within human and porcine epithelial cells was found to be significant, highlighting the importance of high affinity transporters in iron acquisition. We further characterized the *feoB* mutants by microarray experiments, and demonstrated 110 genes to differentially express between the *feoB* mutant and the wild-type strain, with the up-regulation of oxidative stress defense gene *katA* and a non-heme iron protein Cj0012c.

The presence of an extracellular ferric reductase activity was shown from both the wild-type and *feoB* mutant strain, highlighting the existence of a previously uncharacterized system in *C. jejuni*. The *in vitro* studies were supported with an extensive assessment of colonization potential of the *feoB* mutants *in vivo* using three diverse animal models. The *feoB* mutants were consistently affected in their ability to colonize the gastrointestinal tract of the commensal chick colonization model, the infection model rabbit ileal loop and colostrum-deprived neonatal piglets, indicating the phenotype to be an *in vivo* specific defect. This study is the first report of the use of neonatal piglets in the assessing of *C. jejuni* mutants. This extensive characterization of *feoB* mutant suggests the significance of FeoB in iron acquisition and gastrointestinal colonization of animal hosts.

The second objective of characterization of the TonB systems in *C. jejuni* was accomplished by constructing a series of *tonB* mutants. Three single *tonB* mutants and three double *tonB* mutants were constructed. The paucity of alternative tools that can

function in *C. jejuni* resulted in the failure of all our attempts in constructing a triple *tonB* mutant. The *in vitro* characterization of these *tonB* mutants demonstrated the TonBs to be iron regulated. Ferric iron uptake in *C. jejuni* was shown to be proton dependent. Growth promotion assays and $^{55}Fe^{3+}$-enterobactin assays characterized the TonB systems and identified TonBs to perform specific and redundant functions. TonB3 was found to be specifically required for enterobactin utilization, whereas TonB2 and TonB3 were together required for the uptake of hemin and hemoglobin. The three *C. jejuni* isolates tested for their *in vitro* phenotype in our study demonstrated a characteristic pattern in the uptake of different iron sources. This could likely be related to the number of functional TonBs present in the different strains, and their substrate specificity, by virtue of conserved residues in the TonBs. The multiple TonB systems were screened for their role in the colonization of the gastrointestinal tract using the commensal chick model and the infection neonatal piglet colonization model. The *tonB2* mutant was the only mutant to be not affected in chick colonization, whereas, all the other single and double *tonB* mutants were affected in chicks. All the *tonB* mutants were also attenuated in the colonization potential of the neonatal piglets, including the *tonB2* mutant. These *in vivo* colonization data highlighted the importance of having multiple relevant animal models in the thorough characterization of a gene function *in vivo*. Overall, the TonBs were identified to significantly contribute in ferric iron uptake and *in vivo* colonization of the host.

Thus, combining our *in vitro* and *in vivo* characterization of the FeoB and TonB systems, it is clear that both the systems contribute significantly in iron acquisition and colonization of the gastrointestinal tract.

Appendix

Use of Genome-Wide Expression Profiling and Mutagenesis to Study the Intestinal Lifestyle of *Campylobacter jejuni*

Alain Stintzi[1]*, Denver Marlow[2], Kiran Palyada[1], Hemant Naikare[1], Roger Panciera[1], Lisa Whitworth[1] and Cyril Clarke[2]

[1]Department of Veterinary Pathobiology
[2]Department of Physiological Sciences
College of Veterinary Medicine
Oklahoma State University
Stillwater, OK 74078

This paper has been published in the Journal-Infection and Immunity, March 2005, p. 1797-1810.

(*) Author to whom correspondence should be addressed.

Summary of my role in this work

During the course of my doctoral program, I have been a co-author in a recent paper which has been published in the Journal "Infection and Immunity" March 2005, p. 1797-1810.

My contribution towards this paper was my involvement in the *in vivo* competition experiments for screening the *C. jejuni* mutants in their ability to colonize the rabbit ileal loop.

Abstract

Campylobacter jejuni is the most common bacterial cause of diarrhea worldwide. To colonize the gut and cause infection, *C. jejuni* must successfully compete with endogenous microbes for nutrients, resist host defenses, persist in the intestine, and ultimately infect the host. These challenges require the expression of a battery of colonization and virulence determinants. In this study, the intestinal lifestyle of *C. jejuni* was studied using whole genome microarray, mutagenesis, and a rabbit ileal loop model. Genes associated with a wide range of metabolic, morphological, and pathological processes were expressed in vivo. The in vivo transcriptome of *C. jejuni* reflected its oxygen-limited, nutrient-poor, and hyper-osmotic environment. Strikingly, the expression of several *C. jejuni* genes was found to be highly variable between individual rabbits. In particular, differential gene expression suggested that *C. jejuni* extensively remodels its envelope in vivo by differentially expressing its membrane proteins and by modifying its peptidoglycan, and glycosylation composition. Furthermore, mutational analysis of 7 genes, *hspR*, *hrcA*, *spoT*, Cj0571, Cj0178, Cj0341, and *fliD*, revealed an important role for the stringent and heat shock response in gut colonization. Overall, this study provides new insights on the mechanisms of gut colonization, as well as possible strategies employed by *Campylobacter* to resist or evade the host immune responses.

Introduction

Campylobacter is the most common non-viral etiological agent of infectious enteritis in humans and has been implicated in 14.2% of the 76-million food-borne illnesses reported annually in the United States (21). *Campylobacter* infections vary from mild diarrhea to severe abdominal pain (49). Rarely, they result in the development of Guillain-Barré syndrome, which is the primary cause of acute neuromuscular paralysis in the US (49). Because of the extremely high number of cases of food-borne infections reported yearly worldwide, the development of new strategies to fight these infections is urgently needed, and will depend on developing an understanding of host-pathogen interactions. The complete genomic sequence of *C. jejuni* NCTC 11168 was released in 2000 (27) providing new opportunities for the investigation of *Campylobacter* pathogenesis.

Despite the high incidence of *Campylobacter*-mediated diarrhea, the microbial factors that govern gut colonization and pathogenesis are poorly understood in comparison with other enteric pathogens. In a complete infection cycle, *Campylobacter* cells are transferred from contaminated foods to the stomach, the intestinal tract and, finally, to the feces, allowing their transmission to a new host (6, 13). During this stressful journey through the gastrointestinal tract, *Campylobacter* encounters and must adapt to life-threatening environmental conditions, such as the acidic pH of the stomach, the high osmolarity of the gastrointestinal tract, intestinal gases, reactive oxygen and nitrogen compounds, changes in nutrient availability, and low inorganic ion concentrations (13). For successful colonization, *Campylobacter* cells must survive in the intestinal tract, either as free living microorganisms in the mucus layer, attached to the

epithelium, or intracellularly in epithelial cells (6). *Campylobacter* determinants involved in colonization and pathogenesis include flagella, host cell adherence and invasion, and toxin production (6). Non-motile and aflagellated *Campylobacter* were shown to be affected in their ability to colonize the gastrointestinal tract as well as to invade the epithelial cells (25, 56). Many suspected adhesins have been identified, such as lipo-oligosaccharide (20), flagella (56), or surface exposed proteins (CadF, PEB1) (23, 29). Host cell invasion has been extensively studied and is thought to be an important step in *Campylobacter* infection (6). Indeed, biopsies of humans diagnosed with *C. jejuni* enteritis revealed the presence of intra-cellular *Campylobacter* cells (47). Interestingly, toxin production has recently been proposed to modulate the host immune response, allowing the bacteria to escape the immune surveillance (9).

To identify new potential virulence factors, we analyzed *C. jejuni* lifestyle in the gut using microarray technology. The *C. jejuni* NCTC 11168 genome-wide expression profile was assessed during host colonization and pathogenic development, using a mammalian model of gastroenteritis, the rabbit ileal loop model. In addition, mutants were constructed by deleting genes of interest identified by our microarray analysis, and assessed for their ability to survive in the gastrointestinal tract of rabbits.

Materials and Methods

Bacterial strains, plasmids, and preparation of inocula.

The bacterial strains and plasmids used in this work are listed in Table 1. The *Campylobacter jejuni* NCTC 11168 strain was acquired from the National Collection of Type Culture (NCTC, England) in the spring 2000. *Campylobacter* strains were cultured in Mueller-Hinton (MH) medium or on MH agar plates at 37°C in a microaerophilic chamber (Don Whiteley, West Yorkshire, England). Chloramphenicol resistant mutants were maintained on MH medium supplemented with 20 µg/ml of chloramphenicol.

C. jejuni inocula were prepared by microaerobic culture (84% N_2, 5% O_2 and 11% CO_2) in MH medium at 37°C with agitation using a stirrer. The bacterial growth was monitored by measuring the optical density at 600 nm. At early mid-log phase (OD600 of approximately 0.3), the bacterial culture was split in two and one half was used to produce purified total RNA from *C. jejuni* grown in vitro, while the other half was used to inoculate the rabbit ileal loops. Bacteria were collected by centrifugation (10 min, 6000 x g), washed once with sterile PBS buffer, and resuspended in PBS buffer at a concentration of approximately 6.6 x 10^{10} cfu/ml.

For the in vitro growth experiments, the *C. jejuni* wild-type and mutant strains were grown in MH biphasic medium at 37°C under microaerophilic conditions (83% N_2, 4% H_2, 8% O_2 and 5% CO_2).

Rabbit ileal loop model (RIL) and isolation of Campylobacter jejuni total RNA. Rabbits were checked upon arrival to see if they carried *Campylobacter* by taking cloacal swabs for culture. Ileal loops were prepared according to published methods (3, 8). Briefly, New

Zealand White rabbits (<2 kg, female) were anesthetized, a laparotomy was performed, and two 20-cm sections of ileum with intact mesenteric blood supply were ligated per animal. Each loop was inoculated with approximately 10^{11} mid-log phase *Campylobacter jejuni* in 1.5 ml PBS buffer. The size of the inoculum was confirmed by bacterial enumeration on MH agar plates. Loops of two rabbits were injected with sterile PBS buffer and served as control animals. After replacing the intestinal loops in their appropriate position in the abdominal cavity, the abdominal wall and skin were closed in standard fashion and the rabbits were allowed to recover from anesthesia. The rabbits were anesthetized again 24 or 48 hours after the inoculation, the intestinal loops were excised intact, and the animals were then euthanized.

RNA turnover in the samples was quickly stopped by submerging the entire loops into 10 ml of RNAlater solution (Ambion, Austin, TX). The loops were first weighed in order to evaluate fluid accumulation and the contents of each loop as well as the mucus layer then were recovered into 20 ml of a 50% solution of RNAlater in PBS buffer and centrifuged at low speed to remove epithelial cells (5 min at 1,000 x g). Thereafter, *Campylobacter* bacteria were separated from the intestinal microflora by filtration through 0.8 µm filters. *Campylobacter* cells were pelleted by centrifugation and total RNA was isolated using a hot phenol-chloroform protocol, as previously described (40). Traces of genomic DNA were removed by two or three consecutive treatments with DnaseI Amp grade enzyme (Invitrogen, Carlsbad, CA). The absence of contaminating genomic DNA was confirmed by polymerase chain reaction (PCR). RNA was further purified 2 to 5 times using a Qiagen RNAeasy mini kit (Qiagen, Valencia, CA) and the concentration of RNA was determined using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene, OR).

Microarray construction and hybridizations.

DNA microarrays were prepared using PCR amplified fragments of each annotated open reading frame from *C. jejuni* NCTC 11168, as previously described (40, 41). Twenty µg of total RNA from each growth condition (in vitro and in vivo) were converted to cDNA using 2 pmol of *C. jejuni* 3' specific primers (set of 1654 3' primers used for the PCR amplification of *C. jejuni* ORFs) and coupled to monoreactive fluors (Cy3 and Cy5), according to previously described procedures (40).

Data collection and analysis.

Microarray slides were scanned at 532 nm (Cy3) and 635 nm (Cy5) wavelengths with a laser-activated confocal scanner (ScanArray 3000) at 10 µm resolution, generating two TIFF images. Fluorescence intensities of each spot were collected using the GenePix Pro 3.0.5 software (Axon Instruments, Foster City, CA.) after manual optimization of spot registration, and exported to OriginPro 7 spreadsheets (OriginLab Corporation, Northampton, MA). The analysis of the fluorescence data was conducted as follows: (1) The spots were filtered and excluded based on slide abnormalities or low signal (corresponding to spots flagged bad or not found). (2) After background subtraction, all spots with fluorescent mean intensities below three times the standard deviation of the background in both channels were removed from the final data analysis. (3) The fluorescence intensity in each wavelength was $\log_2$ transformed and normalized using locally weighted linear regression (lowess) performed by the MIDAS software (available from TIGR; http://www.tigr.org/software/).

For the microarray analysis, *Campylobacter* RNA was isolated 48 hours post-infection from five RIL rabbits. Each cDNA sample was individually co-hybridized with cDNA obtained from in vitro growth (mid-log phase bacteria) on microarray slides. The microarray hybridization was repeated up to three times depending on the amount of RNA purified from each rabbit, yielding between 2 and 6 measurements per gene per rabbit (each gene was spotted in duplicate on each slide). The microarray data were statistically analyzed using the "Significant Analysis of Microarray (SAM)" algorithm, which was specifically developed for genomic expression data mining (the Microsoft Excel add-in software is available at http://www-stat.stanford.edu/~tibs/SAM/) (46). Briefly, SAM uses the standard deviation of repeated gene expression measurements to assign a score to each gene. It then estimates, for a particular score, a false discovery rate by permutations of the data. This SAM analysis ascertains that genes identified as "differentially expressed" do not arise from a random fluctuation of the large quantity of data generated (46). To identify genes whose expression differed significantly between in vivo and in vitro growth, we performed a one class response analysis by considering the five rabbits as one class. We applied a false discovery rate of 0.11% and a delta value of 0.9. To identify genes with variable expression between rabbits, we performed a multiple classes analysis by treating each rabbit as one class. We applied a false discovery rate threshold of 1.64% and a delta of 0.19. The microarray data of SAM positive genes were extracted into a text output file using the Samster software (available at http://falkow.stanford.edu/whatwedo/software/software.html). Finally, the ratios of the fluorescence intensities of all replicate spots from the hybridization of RNA derived from

each rabbit were averaged and used for further analysis. The data generated by this study are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab.

Campylobacter jejuni mutants construction.

Knockout mutants of *C. jejuni* NCTC 11168 were constructed by independently mutating 6 genes; *hrcA*, Cj0571, *spoT*, *hspR*, Cj0341 and *fliD*. The same inactivation strategy was used for the *hrcA*, Cj0571, *spoT*, and *hspR* mutants. Briefly, the gene to be mutated was amplified by PCR from *C. jejuni* NCTC 11168 chromosomal DNA, which was extracted using a standard protocol (35). The PCR product was cloned into pUC19, using a unique restriction site (Table 2), and deletions of 42 bp, 600 bp, 627 bp, and 72 bp were made by inverse PCR in *hrcA*, Cj0571, *spoT*, and *hspR*, respectively. The chloramphenicol resistance cassette (Cam^r) was PCR amplified from pRY111 (55) using primers with appropriate restriction sites and cloned into the deletion site. Specific primers used for the first PCR amplification and the following inverse PCR are listed in Table 2. Recombinant plasmids carrying the Cam^r gene in the same orientation as the genes of interest were selected by DNA sequencing and transformed into *C. jejuni* NCTC 11168 using standard protocols (55). Transformants were identified on MH agar plates containing 20 µg/ml of chloramphenicol. The identity of the mutants was confirmed by PCR analysis using a combination of primer sets annealing within the mutated gene and the Cam^r gene.

The Cj0341 and *fliD* mutants were isolated from a library of random mutants generated using the EZ::TN^{TM} $pMOD^{TM}$-3<R6Kγori/MCS> transposon (Epicentre), in which the Cam^r cassette from pRY111 has been cloned (Stintzi, unpublished data). The mutant library was constructed following the manufacturer's recommendation, and the Cj0341

and *fliD* mutants were identified during the first trial to assess the randomness of the library. The insertion site of the transposon in the Cj0341 and *fliD* mutants was identified using a single primer PCR procedure and DNA sequencing of the resulting amplification product, as described by others (16). The Cj0341 and fliD mutations were confirmed by PCR amplification using a primer that anneals within the mutated gene and another primer that anneals within the Cam^r gene.

In vivo survival assays using a pool of mutants.

The ability of the *C. jejuni* mutants to survive within the rabbit ileal loops was assessed. Each mutant was grown individually in MH broth to mid-log phase, harvested by centrifugation (10 min, 6000 x g), washed and resuspended in PBS buffer. The mutants were mixed to constitute the input pool by combining 5 x 10^9 cfu of each mutant with 7.5 x 10^{10} cfu of the wild-type strain, *C. jejuni* NCTC 11168, in 5 ml of PBS buffer. One ml of this suspension was used to prepare genomic DNA following standard protocols. The remaining bacterial suspension was used to equally inoculate 4 ileal loops from 2 rabbits (1 ml per loop). At 48 hours post-infection, loop contents were harvested in PBS buffer and centrifuged at low speed to remove debris and epithelial cells. The supernatant was immediately processed to prepare genomic DNA, constituting the recovered pool of genomic DNA. Each loop was processed individually.

The difference in relative abundance of each mutant between the input and recovered pools was evaluated by quantitative PCR, which was performed using an ABI Prism 7700 DNA analyzer (Applied Biosystems, Foster City, CA) and the QuantiTect™ SYBR® Green PCR kit (Qiagen, Valencia, CA), according to the following protocol: 500 ng of

genomic DNA was added to 25 µl of 2xQuantiTect SYBR Green PCR solution and 0.3 µM of each specific primer in a 50 µl final volume. The HotStar Taq DNA polymerase was activated by heating the reaction at 95°C for 15 min. PCR amplification was performed by 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s. The specificity of the PCR reaction was confirmed by melting curve analysis of the PCR product following the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Genomic DNA of each mutant was specifically amplified from both pools by using a combination of two primers, with one of them annealing within the mutated gene and the other within the chloramphenicol resistant cassette. The sequences of the specific primer sets are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab. The relative abundance of each mutant was then normalized to the DNA pool by using the *cydA* or *argD* genes. The competitive ratio of the relative abundance of each mutant between the input and recovered pools was obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as recommended by Applied Biosystems. The abundance of each mutant was assessed twice per loop and the mean C_T value for each ileal loop was used for further analysis. The C_T value corresponds to the PCR threshold cycle at which the fluorescence detected is significantly higher than the baseline value. The ratio of the mutant in the input to the recovered pool was calculated as follows: ratio input/recovered = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T,\ recovered} - \Delta C_{T,\ input}$, and $\Delta C_{T,\ recovered\ or\ input}$ is obtained by subtracting the mean C_T value of the specific gene from the mean C_T value of the reference gene (*cydA* or *argD*) in the genomic DNA from the input or recovered pool. Given that the wild-type strain was inoculated at a higher level than the mutants, the population as a whole (the mutants

plus the wild-type strain) should have a growth similar to the wild-type strain alone. The same assumption is usually made for the analysis of data generated by signature tag mutagenesis (10). Consequently, the normalized competitive ratio input/recovered of *C. jejuni* NCTC 11168 should be approximately equal to 1. A competitive ratio input/recovered above 1 indicated that the mutant was attenuated in vivo, while a competitive ratio below 1 indicated that the mutant survived better in vivo than the wild-type. The data was statistically analyzed using the Student *t*-test and a *P* value below 0.01 was considered significant.

In vitro and in vivo competition experiments.

C. jejuni wild-type and mutant strains were grown in biphasic MH medium to mid-log phase, centrifuged and resuspended in PBS buffer to $OD_{600} \approx 1.8$ (approximately 10^{10} cfu/ml). Two milliliter (each) of the wild-type and the mutant strains were mixed at a 1 to 1 ratio. Four ileal loops (from 2 rabbits) were injected (each) with 1 ml of this suspension. The initial 1:1 mixture of mutant and wild-type strains was confirmed by plating serial dilutions of this mixed culture on MH agar with and without chloramphenicol (20 μg/ml). At 48 hours post-infection, the loops were recovered as described above. Their content as well as the mucus layer were collected and homogenized in 10 ml of PBS buffer. Serial dilutions of bacteria recovered from each rabbit's loop were plated on karmali-agar plates (*Campylobacter* agar base (Oxoid CM935) supplemented with the *Campylobacter* selective karmali supplements (Oxoid SR167E)) and karmali-agar plates containing chloramphenicol (20 μg/ml). Plates were incubated at 37°C for 3 days before the colonies were counted. The titer of the mutant

was obtained from the CFU recovered on karmali-agar plates containing chloramphenicol, and the titer of the wild-type bacteria was calculated by subtracting the number of mutants to the total number of bacteria recovered on karamali-agar plates without antibiotic. Finally, the in vivo competitive index was calculated for each loop and corresponds to the ratio of the mutant to the wild-type strain.

For the in vitro competition assays, overnight cultures of the wild-type and mutant strains were mixed in a 1:1 suspension into fresh MH medium. This suspension was used to inoculate three replicate biphasic MH cultures. The bacterial growth was monitored by measuring the OD_{600} over time. The titer of each strain was determined in the inoculum, and at early stationary phase, by plating on MH-agar and MH-agar containing chloramphenicol (20 μg/ml). The in vitro competitive index was calculated for three independent growth experiments and is defined as the ratio of mutant to wild-type.

A Student's *t* test was used to statistically analyze the data from the in vivo and in vitro competition assays.

*Real-time quantitative RT-PCR analysis***.**

The relative expression of 9 genes (*flgE2*, Cj0178, *katA*, *spoT*, *ahpC*, *fliD*, Cj0571, *cydA*, and Cj0366) was confirmed by real-time quantitative RT-PCR, as previously described (40), using the QuantiTectTM SYBR Green RT-PCR kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. The relative expression of each gene was normalized to either the 16S or 23S RNA, and the extent of its induction was obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as described above. The

primers used are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab.

Necropsy and histopathology.

Four rabbits were used to evaluate the pathological changes in the RIL model caused by *C. jejuni* NCTC 11168. The loops were prepared as described above for the transcriptional profiling experiments. The loops from two rabbits were inoculated with 1 x 10^{11} cfu of mid-log phase *C. jejuni* NCTC 11168, while the loops of the two other rabbits were injected with sterile PBS buffer. At 48 hours post-infection, the rabbits were anesthetized, the loops were recovered, and the animals were then euthanized with an overdose of Beuthanasia D (>0.25 ml/kg of body weight). A 0.5 cm middle section of each loop was immediately excised, flushed with formalin, linearly opened, placed flat on a card, and fixed in buffered 10% formalin, embedded in paraffin, section at 5 μm and stained with hematoxylin and eosin. Specimens were examined for evidence of inflammation, villus epithelial cell attenuation and crypt dilatation or hyperplasia.

Results and Discussion

The rabbit ileal loop (RIL) model for campylobacteriosis.

This animal model was initially chosen because of its documented ability to accurately model the histopathological lesions associated with human *Campylobacter* gastroenteritis (8). In addition, *Campylobacter* cells can be collected in a number sufficient for the investigation of in vivo genome-wide transcripts abundance. The model was created by surgical ligation of 20 cm sections of ileum, resulting in the cessation of the normal peristaltic flux, thereby facilitating *Campylobacter* gut colonization. The strain of *C. jejuni* NCTC 11168 used in our study is helically shaped, fully motile, and colonizes the gastrointestinal tract of chicks (26). Therefore, this strain is phenotypically different from the sequenced *C. jejuni* NCTC 11168 strain recently described by Gaynor et al. (11), which was described to be straight rod-shaped, non-motile, and a poor colonizer of chicks. To explore the feasibility of this model to study *Campylobacter* lifestyle in the gut by transcriptome profiling, we undertook a pilot study involving 4 rabbits: Rabbit intestinal loops were sampled at 24 or 48 hours post-inoculation (using 2 rabbits per time point). To note, the *C. jejuni* strain was passaged three times in vitro before its inoculation in the ileal loops. *C. jejuni* NCTC 11168 colonized the rabbit gut at bacterial concentrations of 10^5 cfu/loop and 10^7 - 10^9 cfu/loop, at 24 and 48 hours post-inoculation, respectively. The initial decrease in bacterial population from 10^{11} to 10^5 cfu per loop during the first 24 hours after inoculation reflects the challenges of surviving in a hostile environment, while the subsequent bacterial growth from 10^5 to 10^9 cfu/loop at 48 hours suggests the successful adaptation and colonization of *C.jejuni* in the rabbit intestinal

tract. Considering that 10^5 cells would not generate sufficient amounts of RNA to perform microarray hybridization, we decided to harvest the bacterial cells at 48 hours post-infection in the present study.

Intestinal distension resulting from accumulation of gas and fluid (the first signs of diarrhea) was qualitatively observed at 48 hours post-infection in all infected rabbits but not in the control animals injected with the PBS buffer. Two infected rabbits and two control animals were used to evaluate pathological changes. Fluid accumulation was quantitatively estimated by weighing the intestinal content of the 8 loops, which indicated an increase of 0.09 ± 0.05 g content/g of ileal tissue in the infected loops compared with control loops. This difference was found to be statistically significant ($P < 0.05$ using a paired *t*-test). In contrast to the observations of Everest *et al*. (8), histopathological analysis of the ileal tissues did not reveal any severe pathology. This lack of damages likely reflects the inability of *C. jejuni* NCTC 11168 to invade epithelial cells or to exert morphologically evident cytotoxic effects on intestinal epithelial cells. In fact, the strain of *C. jejuni* NCTC 11168 used in our study is poorly invasive into human epithelial INT407 cells (30). Consequently, the transcriptome profile presented in this work reflects non-invasive *Campylobacter* lifestyle in the intestine during survival, colonization and the initial stages of pathogenesis.

In vivo expression profiling validation.

While microarrays provide a powerful approach for the investigation of gene expression, the performance of these expression studies *in vivo* is technically challenging. To date, expression profiling experiments have been limited mainly to in vitro environments. In

the present study, in vivo colonization of the intestinal tract by *C. jejuni* was investigated by conducting transcriptional expression profiling experiments during growth and survival within the natural gut environment. We utilized microarrays containing spotted PCR products representing approximately 98% of the annotated open reading frames of *C. jejuni* NCTC 11168 (40). The challenge of recovering intact *C. jejuni* mRNA from the intestine to ensure acquisition of an accurate and specific transcriptome profile was addressed by excising the entire intestinal loops and immediately submerging them in RNA stabilization solution to block RNA turnover. In order to minimize RNA degradation and/or changes in the gene expression level, loops were immediately processed for RNA extraction and quantitative histopathological traits were not recorded. The content of each loop, including the mucus layer, was recovered in RNA stabilization solution, and *C. jejuni* was purified by filtration through 0.8 µm filters. This physical separation removed most of the endogenous microflora; more than 80% of the bacterial population was estimated to be constituted of *C. jejuni*. The yield of RNA recovered was between 12 to 55 µg per loop. The total RNA extracted from each rabbit's two loops were combined. Twenty µg of RNA was reverse transcribed using *C. jejuni* specific 3' end primers and fluorescently labeled with the Cy5 dye which fluoresces red. The relative abundance of transcripts was monitored by competitive hybridization with RNA extracted from bacteria grown in vitro to mid-log phase and labeled with the green fluorescent Cy3 dye. To address any potential cross-hybridization with RNA extracted from the remaining natural intestinal microflora, RNA was also purified from ileal loops of rabbits which had been injected with PBS buffer only. The yield of total RNA purified from the uninfected loops was between 1 and 3 µg per loop. The total RNA harvested

from two uninfected loops were combined, reversed transcribed, labeled with Cy5 and hybridized to the *C. jejuni* microarray. As shown in Figure 1, this RNA did not cross-hybridize with genes from *C. jejuni*. In addition to physical enrichment of *C. jejuni* by filtration, the use of 3' specific primers to synthesize cDNA further enhanced the specificity of the assay. A similar approach was employed by Talaalt and coworkers to amplify mycobacterial RNA from a mixture containing mammalian RNA (43).

Global gene expression analysis and validations

C. jejuni NCTC 11168 was inoculated into 5 rabbits and colonized the loops of these rabbits at a bacterial concentration of 3.10^8 cfu/loop (rabbit 1), 2.10^8 cfu/loop (rabbit 2), 5.10^8 cfu/loop (rabbit 3), 4.10^7 cfu (rabbit 4), and 4.10^8 cfu/loop (rabbit 5). *Campylobacter* RNA samples were extracted from each rabbit 48 hours post-infection and individually hybridized to the microarray slides up to three times, depending on the amount of RNA purified from each loop. Specifically, rabbits 1, 4 and 5 yielded two measurements per gene, rabbit 2 yielded six measurements per gene, and rabbit 3 yielded four measurements per gene. The data were quantified, normalized, and reported as the ratio of gene expression of *C. jejuni* grown in the rabbits to that of *C. jejuni* grown in vitro. To limit the number of genes falsely identified as differentially expressed, we performed a statistical procedure. This test consisted in applying the significance analysis of microarray (SAM) algorithm to our microarray data. This statistical method has been shown to be more reliable than a standard *t* test or the use of a fold change threshold, and is relatively conservative in declaring a significant change in gene expression (46). A one class response SAM analysis, using the five rabbits as one group, identified 348 genes as

being differentially expressed between in vivo and in vitro growth with a false discovery rate of 0.11%. All SAM selected genes exhibit expression ratios greater than 1.5. As demonstrated in our previous study using the same microarray platform, a 1.5 fold differential expression is technically and biologically significant (40). This *Campylobacter* microarray platform has previously been shown to generate data with a high level of concordance with quantitative RT-PCR (40). However, in order to address the reliability of the microarray data generated in this study, the change in transcript abundance in rabbit 4 between in vitro and in vivo growth was confirmed for 9 genes (*flgE2*, Cj0178, *katA*, *spoT*, *ahpC*, *fliD*, Cj0571, *cydA*, and Cj0366) by real-time quantitative RT-PCR. The *flgE2* gene was found to be 4 fold up-regulated, Cj0178 was 200 fold up-regulated, *katA* was 130 fold up-regulated, *spoT* was 61 fold up-regulated, *ahpC* was 4 fold up-regulated, *fliD* was 5 fold down-regulated, Cj0571 was 2.5 fold up-regulated, *cydA* was 350 fold up-regulated and Cj0366 was 300 fold up-regulated. Similarly to our previous study, while the quantitative RT-PCR confirmed the trend in differential gene expression observed with the microarray analysis, a quantitative difference in the fold change was observed between these two technologies. This difference reflects a lower dynamic range for the microarray experiments as compared to quantitative real-time RT-PCR, as previously reported by others (53). Notably, very few genes were found to be differentially regulated more than 20 fold by the microarray analysis while the real-time RT-PCR found several genes up-regulated more than 100 fold. This observation highlights the semi-quantitative nature of microarray experiments and the low dynamic range of this technology (4). Furthermore, this technical limitation appears to be amplified in situations where a gene exhibits a very low expression level

under only one of the growth condition, which is the case of in vivo genome-wide expression analysis. As a consequence, the fold change in gene expression presented in this study should be significantly underestimated. Nevertheless, differentially expressed genes were readily identified by statistical analysis. Overall, 185 genes were found to be induced in vivo in all five rabbits. Among them, 177 exhibited more than two-fold differential expression with 91 of them showing more than 4-fold differential expression. Of the 199 genes found to be repressed in vivo, the expression level of 153 genes was reduced 2 fold and the expression level of 32 genes was reduced more than 4 fold.

Multiple class response SAM analysis (considering each rabbit as one group) as well as two class unpaired data SAM analysis (considering each rabbit as one group and comparing each rabbit with each other) revealed some gene expression variability between rabbits. Multiple class response SAM analysis identified 170 genes differentially expressed between rabbits with a false discovery rate of 1.64%. Importantly, very few genes were found to be antagonistically expressed between rabbits. Indeed, the trend of differential expression remained essentially the same, while only the amplitude of change in transcript abundance varied. To confirm the variability of gene expression between rabbits and to rule out the possibility of intrinsic noise, we compared the expression measurements of these 170 genes within each rabbit and between rabbits (using the microarray data from rabbits 2 and 3). A high level of concordance with a correlation coefficient higher than 0.9 was obtained between replicate microarray hybridizations of RNA isolated from the same rabbit (Figure 2, panels A and B), whereas a very weak correlation was observed between hybridizations of RNA samples originating from two different rabbits (Figure 2, panel C). The variability of gene expression between rabbits

was further confirmed by quantitative real-time RT-PCR for *flgE2* and Cj0178, which encode for the flagellar hook subunit protein and a putative outer membrane ferric-siderophore receptor, respectively. These were found to be differentially expressed between rabbits 3 and 4. The microarray analysis indicated that the *flgE2* gene was over-expressed in rabbit 4 and down-regulated in rabbit 3, while the expression of Cj0178 was essentially unaffected in rabbit 3 and up-regulated in rabbit 4. By using the same RNA preparation as the one used for the microarray hybridization, quantitative RT-PCR confirmed the differential expression of both genes. The expression of *flgE2* was found to be down-regulated approximately 70-fold in rabbit 3, and up-regulated 4-fold in rabbit 4, compared with in vitro growth. Cj0178 was found to be equally expressed in rabbit 3 and over-expressed 200-fold in rabbit 4 relative to in vitro growth.

The observed variability in gene expression patterns is unclear but likely reflects both physiological and intrinsic variations in the rabbits. This hypothesis is in agreement with the observed difference in colonization level (1 log) and the variation in the amount of fluid accumulation (±55%) between rabbits. Obviously, the gastrointestinal environment cannot be controlled and is likely to vary from one rabbit to another, leading to variations in *C. jejuni* colonization and gene expression profiles. In addition, it is unknown if the rabbits used in this study had previously encountered *C. jejuni*. If it was the case, an immune response would likely take effect by 48 hours post-infection and might also result in the observed gene expression variability. Recently, Boyce *et al.* reported the genome-wide expression profile of *Pasteurella multocida* recovered from blood of infected chickens 20 hours after inoculation (2). Although blood has questionable pathological relevance because it is not the site of infection of *P. multocida*, similarly to

our study the authors observed a variable bacterial gene expression profile between infected hosts (2). More recently, Xu *et al.* characterized the transcriptome of *Vibrio cholerae* during intestinal growth 8 hours post-infection using the rabbit ileal loop model (54). In contrast to our study, *V. cholerae* gene expression was similar in the three rabbits tested. All together, these data highlight the complexity of studying genome-wide gene expression in vivo.

Campylobacter **lifestyle in the gut.**

Overall, the expression of 482 genes was found to be significantly altered in vivo. Based on their expression profiles, genes can be grouped into two major categories: (1) genes exhibiting similar differential expression in all 5 rabbits tested (348 genes, Figure 3); and (2) genes with variable expression between rabbits (170 genes, Figure 4). It should be noticed that 36 genes belong to both categories. These genes exhibit similar expression alteration in vivo in all five rabbits but different fold change amplitudes between rabbits.

To elucidate further the intestinal lifestyle of *Campylobacter*, we grouped genes by functional annotations and mapped their expression profiles to all known biological processes, thus allowing the investigation of the overall physiological status of *C. jejuni* grown in vivo. This approach revealed the involvement of a wide range of metabolic, morphological and pathological processes (Figures 3 and 4). Figures 3 and 4 list only the genes found to be significantly differentially expressed between in vivo and in vitro growth by SAM analysis. However, a biological process was considered to play a role in *Campylobacter* physiology in the intestine when the constituting genes were found to be either up-regulated or equally expressed in vivo compared to in vitro growth.

Energy and central intermediary metabolism

The in vivo transcriptome pattern of *C. jejuni* was consistent with the oxygen-limited environment found in the intestine. The expression of genes encoding for the key enzymes in the oxidative phosphorylation pathway was decreased dramatically in all 5 rabbits. These genes encode for NADH dehydrogenase (*nuoG, nuoL* and *nuoH*), and succinate dehydrogenase (*sdhABC*). Recently, fumarate, nitrate, nitrite, and N- or O-oxides have been shown to constitute alternative terminal electron acceptors, allowing *C. jejuni* to carry out respiration under oxygen-restricted conditions in vitro (36). However, the genes encoding for the reductases involved in this alternative respiratory pathway were all down-regulated in vivo. In contrast, the genes encoding for the cytochrome bd oxidase (*cydAB*) were expressed in vivo but not or only slightly expressed in vitro. The differential expression of the *cydA* gene was confirmed by quantitative real-time PCR. The expression of *cydA* was found to be 350 fold higher in vivo compared to in vitro growth. Although the CydAB oxidase catalyses the oxidation of menaquinone using oxygen as an electron acceptor, this enzymatic complex has been shown in *E. coli* to possess a high affinity for oxygen, allowing the bacterium to carry out respiration under limited oxygen tension (5). In addition, the expression of *E. coli cydAB* is known to be induced under limiting oxygen conditions (5). Similarly, the CydAB complex could facilitate *C. jejuni* respiration in the oxygen-limited environment of the intestine. Interestingly, formate dehydrogenase (encoded by *fdhABCD*) was the only enzyme identified by the microarray analysis to be over or equally expressed in vivo relative to in vitro and capable of transferring electrons to the menaquinone pool. Other genes

encoding enzymes with similar activity were found to be down-regulated in vivo. FdhABCD enzyme participates in the respiratory chain of many bacterial species, enabling these organisms to respire using formate as an alternative terminal electron donor under anaerobic conditions (33). In *C. jejuni*, the formate dehydrogenase, together with the CydAB complex, could allow the bacterium to carry on oxygen respiration even under extreme oxygen limited conditions.

The expression of the genes encoding enzymes involved in gluconeogenesis, the citric acid cycle, and the pentose phosphate pathway, were all down-regulated in vivo, except for fructose bi-phosphate aldolase (*fba*). Down-regulation of these genes is consistent with the oxygen deprived intestinal environment and the up-regulation of the carbon storage regulator, *csrA*. In *E. coli*, CsrA has been shown to repress the expression of genes involved in glycogen catabolism, gluconeogenesis, glycolysis, and motility (34). This enzyme likely performs a similar function in *C. jejuni*.

Macromolecular synthesis and processing

Genes encoding proteins involved in the synthesis and modification of macromolecules, in particular the ribosomal proteins (with the exception of the *rpsA* gene) and aminoacyl tRNA synthetases, were among the most highly up-regulated in vivo. The significance of this contradictory expression of *rpsA* (which encodes the ribosomal protein S1) and other genes from the same functional group is puzzling and requires confirmation by an alternative method and further investigation. In *E. coli*, the ribosomal protein S1 has been shown to be essential for cell viability, to promote the efficiency of translation, and to act as a repressor for its own synthesis (37). Depletion of the protein S1 resulted in a

stringent response consistent with amino acid starvation and an increased production of ppGpp (37). Therefore, the down-regulation of the *rpsA* expression would suggest the induction of a stringent response in *C. jejuni* during intestinal colonization.

Biosynthesis of cofactors

Another group of genes expressed in vivo encodes proteins involved in the biosynthesis of the cofactors, biotin (*bioABCD*), riboflavin (*ribADFH*), thiamine (*thiCDEGHJL*), pantothenate (*panBC*), coenzyme A (*accB* and *acs*) and folic acid (*folCD*). These genes were found to be either up-regulated or equally expressed in vivo relative to in vitro growth (with the exception of two genes, *thiG* and *D*, from the thiamine biosynthetic pathway, which were found to be down-regulated). The expression of these genes suggests that these cofactors are unavailable in the intestine. As a consequence, and because biotin, riboflavin, thiamine and pantothenate are produced only by microbes and higher plants, these biosynthetic pathways could constitute an ideal target for drug development. This evidence that biotin is unavailable in the intestine is corroborated by the up-regulation of *V. cholerae* biotin biosynthetic genes during intra-intestinal growth (54), and by the inability of a *V. cholerae* biotin biosynthesis mutant to colonize the gastrointestinal tract of mice (22).

Virulence and colonization determinants

Suspected virulence and colonization factors of *Campylobacter* include motility and chemotaxis, host cell adherence and invasion, toxin production, lipo-oligosaccharide and surface structure biosynthesis, oxidative stress defense, iron acquisition and heat shock

response (49). In contrast to genes encoding proteins involved in general metabolism or bacterial physiology, the expression of many genes related to virulence and/or colonization factors were highly variable among infected rabbits. The most notable among genes with flexible expression were those coding for proteins involved in flagellum biosynthesis. Motility is known to be an essential requirement for *C. jejuni* to colonize the host gut and ultimately cause disease (49). Considering that the flagellin subunit is the immunodominant antigen recognized during human or animal infection, it is assumed that the gene encoding this protein is expressed in vivo (28). However, our microarray data suggest that there is considerable inter-animal expression variability among genes belonging to the flagellum locus. Most of the genes belonging to the flagellum locus were found to be down-regulated in 4 rabbits (and at a different level), while they were slightly up-regulated in one rabbit. This variability may allow the bacterium to evade the host immune system by shutting down flagellum production once colonization is accomplished. In support of this hypothesis, *C. jejuni* flagella have recently been proposed to be necessary for passage through the gastrointestinal tract of chickens, but not for persistence in the chicken's ceacum (51). A similar effect on the expression of flagellar genes was also recently demonstrated in *Salmonella enterica* during macrophage intracellular growth (7).

Another functional category of genes expressed in vivo relates to iron-responsive genes which encode proteins involved in iron metabolism and oxidative stress defense. Several of the genes encoding iron acquisition systems were found to be either up-regulated or equally expressed between in vivo and in vitro growth. These genes code for a putative ferric-siderophore transporter system (Cj0178 and Cj0173c-Cj0175c), a putative iron

transporter (p19 and Cj1658), and the three TonB-ExbB-ExbD energy transducing complexes. The genes encoding a putative heme outer-membrane transporter (ChuABCD) were found to be only slightly expressed in vivo, suggesting that heme does not constitute the main iron source in the gut. The genes encoding the components of the ferric-enterobactin uptake permease (*ceuBCDE*) were found to be up-regulated in vivo, however the *cfrA* gene encoding the ferric-enterobactin receptor appeared to not be expressed. As a microaerophilic bacterium, *C. jejuni* must deal with free oxygen radicals and other reactive molecules generated by normal aerobic metabolism and host defenses against microbial attack (49). The expression of most genes known to be associated with *C. jejuni* oxidative stress response was found to be increased during gastrointestinal growth (49). To note, the up-regulation of these genes is in agreement with an iron limited environment. Theses genes include *sodB* (superoxide dismutase), *ahpC* (alkyl hydroperoxide reductase), *tpx* (probable thiol peroxidase) and *katA* (catalase). Consistent with the expression of these genes in vivo, a mutation of *C. coli sodB* has been reported to impede colonization of chick gut (31). Furthermore, a mutation in *katA* sensitizes *C. jejuni* to hydrogen peroxide and reduces its intracellular survival in macrophages (49). In *H. pylori*, the catalase KatA has been shown to be required for persistent colonization in the mouse model (14). These results highlight the iron-restricted conditions in the rabbit intestine. The importance of iron metabolism for successful host colonization has been established for most pathogens (32), and should also be an essential factor for *C. jejuni* colonization in the intestine. Indeed, a *fur* mutant of *C. jejuni*, as well as a *cfrA*, *ceuE* and Cj0178 mutants, were recently shown to be significantly affected in their ability to colonize the gastrointestinal tract of chicks (26).

Interestingly, *C. jejuni* possesses a system of general protein glycosylation, which has been proposed recently to play an important role in *C. jejuni* pathogenesis (42). Indeed, mutation of genes encoding the glycosyltransferases, *pglB* (also named *wlaF*) and *pglE* (also named *wlaK*), affected their ability to adhere to and invade human intestinal cells, as well as to colonize the gastrointestinal tract of mice (42). In our study, the expression of three genes, *pglB*, *pglE* and *pglG*, which belong to this functional category, was found to varied between growth conditions. The expression of *pglB* and *pglG* was highly induced in vivo (in 4 rabbits) while the *pglE* expression was either similar or repressed in vivo compared with in vitro growth. The expression of the other genes from the glycosylation cluster (*pglH*, *pglA*, *wlaJ*, and *pglF*) was found to be similar in both growth conditions. It is not clear why expression of the genes belonging to the same biological pathway varied. However, a similar difference in expression between the *pgl* genes was observed previously in *Campylobacter* in response to temperature up-shift (40) and iron starvation (26). Considering the absence of data on the functional role of each *pgl* gene in protein glycosylation, the significance of their expression profiles is difficult to assess.

The genes encoding the recently discovered multidrug efflux pump in *C. jejuni*, *cmeABC*, were found to be highly up-regulated in vivo. Expression of *cmeB* was confirmed by real time RT-PCR to be up-regulated by approximately 300-fold in vivo compared with in vitro growth. This tripartite multidrug efflux transporter is composed of an outer membrane protein, CmeC (Cj0367c), a periplasmic fusion protein, CmeA (Cj0365c), and an inner membrane efflux transporter, CmeB (Cj0366c) (18). Interestingly, this efflux system has been shown to contribute greatly to bile resistance and to be required for the colonization of the chick's gastrointestinal tract (18, 19). Given the presence of a high

concentration of bile salts in the gut, the up-regulation of these genes could contribute significantly to the survival of *Campylobacter* in the host by allowing the bacterium to resist the harmful effects of these salts.

Another important functional set of genes with variable expression between rabbits encodes for proteins involved in peptidoglycan biosynthesis. Specifically, *murB* (a putative UDP-N-acetylenolpyruvoylglucosaminereductase*),* *murC (*UDP-N-acetylmuramate-alanine ligase*),* and *pbpC (*penicillin-binding protein*)* were among the genes from this category that were the most differentially regulated. The differential expression of this category of genes suggests that there may be a modification of the murein sacculus in vivo, probably in response to the high osmolarity of the intestinal environment. Likewise, *Staphylococcus aureus* modifies its peptidoglycan layer under conditions of high osmolarity (50). While the activation of genes involved in peptidoglycan synthesis may constitute a repair mechanism necessary for the bacterial adaptation to environmental stress, the modification of the peptidoglycan structure may have broad implications for the stiffness and elasticity of the cell surface, thereby conditioning the bacterium to its ecological niche.

Among the other genes annotated or previously characterized as virulence- or colonization-associated factors, Cj1279c (putative fibronectin domain-containing lipoprotein) and several genes involved in the heat shock response were found to be significantly up-regulated in vivo. Heat shock proteins are induced in response to various stresses and act by repairing and preventing damage caused by the accumulation of unfolded proteins. The importance of the heat shock response for *Campylobacter* intestinal tract colonization has previously been demonstrated (49). Genes identified as

heat shock proteins (40) and induced in vivo include *clpB* (ATP-dependent CLP protease ATP-binding subunit), *dnaK* (heat shock protein), *grpE* (heat shock protein), *hrcA* (putative heat shock regulator), and *htpG* (hsp90 family heat shock protein). The up-regulation of these genes, together with the previous identification of ClpB as a B-cell antigen in human disease (45), suggests that these proteins play an important role in bacterial growth within the gastrointestinal tract.

Mutational analysis.

In order to study further the *C. jejuni* lifestyle in the intestinal tract of rabbits, we constructed knockout mutations of genes identified by our microarray analysis and investigated the ability of these mutants to survive in the rabbit intestinal loop by comparison with the parent strain, *C. jejuni* NCTC 11168. Seven genes were selected and individually mutated. The main goal of this mutational analysis was to disrupt physiological functions that appeared to be important for the colonization of the ileal loop. In particular, the microarray data suggested an important role for the genes involved in the heat shock response, the stringent response, the iron metabolism, and the biogenesis of the flagellum in the intestinal lifestyle of *C. jejuni*. Therefore, the heat shock response was disrupted by mutagenesis of its two transcriptional regulators *hrcA* and *hspR*. The stringent response was disrupted by mutagenesis of the *spoT* gene which encodes the guanosine-3',5'-bis(diphosphate)3'-pyrophosphohydrolase. The iron metabolism was disrupted by mutagenesis of the Cj0178 gene which encodes a ferric-siderophore outer-membrane receptor. This gene has been previously shown to be induced under iron restriction and is highly up-regulated in vivo. The flagellum

biogenesis was disrupted by mutagenesis of the *fliD* gene which encodes for the flagellar hook-associated protein. A mutant in the *fliD* gene has been shown to be non-motile (12). The *fliD* gene was found to be down-regulated in all 5 rabbits. Interestingly, among the genes encoding transcriptional regulators, Cj0571 was the only one from this category found to be significantly up-regulated in all 5 rabbits by the microarray analysis, suggesting an important role for this protein in vivo. Consequently, this gene was also mutated. Finally, a mutant into Cj0341 was chosen to be tested in the ileal model as an experimental control. The expression of this gene was found to be off under in vivo and in vitro growth conditions. Therefore, this mutant should not be affected in its ability to colonize the ileal loop.

The 7 mutants and the parent strain were pooled together and inoculated into 4 ileal loops constructed in 2 different rabbits. After 48 hours post-inoculation, the ileal contents were recovered and directly processed for chromosomal DNA purification. Then, the relative amount of each mutant was evaluated by quantitative real-time PCR, as described in the Materials and Methods section. The competitive ratio of the number of cells at the time of the inoculation (in the input pool) to the number of cells recovered 48 hours post-inoculation was normalized to the entire bacterial population in the pool for each mutant (Figure 5) so that the competitive ratio of the whole population is equal to 1. Considering that the wild-type strain was present in excess, compared with each mutant in the input pool, and assuming that it represents the major proportion of the population in the recovered pool, it should have a competitive ratio input/recovered equal to approximately 1. Therefore, any deviation of the competitive ratio from a value of 1 would indicate an effect of the mutation on the survival ability of *C. jejuni* in the rabbit ileal loop. As

expected, the competitive ratio of the Cj0341 mutant was 0.9, indicating that this strain colonizes the ileal loop as well as the wild type. Of the 6 other mutants, 1 was unaffected (Cj0571), 1 had an advantage over the others with respect to survival in and colonization of the rabbit ileal loop (*fliD*; $P<0.003$), and 4 were significantly attenuated (*hrcA*, Cj0178, *spoT* and *hspR*; $P\leq0.002$). The *spoT* and *hspR* mutated strains were the most affected mutants, while the *hrcA* and Cj0178 mutants were only slightly attenuated. Because mutants are out-competed by many other strains during a mixed infection, the in vivo phenotype of the affected mutants was confirmed in a 1 to 1 competition assay. Each mutant was independently mixed with the wild-type strain in equal numbers and injected into 4 ileal loops (constructed in two rabbits). The 1 to 1 ratio of the inocula was confirmed by cfu determination. Forty-eight hours post-infection, the loop contents were plated on selective medium for bacterial enumeration. Then, the competitive index was calculated as the ratio of the mutant to the wild type strain recovered from each ileal loop. As shown in Figure 6 (panel A), 3 out of the 5 mutants were confirmed to be statistically affected in their colonization ability (with a $p \leq 10^{-4}$). In order to determine whether the colonization phenotype of these mutants was specific for in vivo growth, an in vitro competition assay was performed. An equal amount of each mutant and wild-type strain was mixed in MH broth. The cultures were incubated at 37°C until late log phase (~30 hours), after which serial dilutions were plated on MH-agar with or without chloramphenicol. These experiments were performed in triplicate. The in vitro competitive index was determined as described for the in vivo competition assay (Figure 6, panel B). Four of the 5 mutants were found to be statistically affected in their ability to out-compete the wild-type strain during in vitro growth. Finally, in order to determine

whether the in vitro growth defect was caused by the competition with the wild-type strain, the growth kinetic of each mutant was independently determined (Figure 7). All five mutants were found to have a growth defect in vitro, with the *spoT*, *hspR*, and *fliD* mutants being the most affected.

The competitive index of the *hspR* mutant in vitro and in vivo was $1.5.10^{-2}$ and 7.10^{-5} respectively. While this mutant is affected in vitro, the 200 fold difference between the in vitro and in vivo competitive indexes indicates a significant in vivo specific growth defect. The attenuation of the *hspR* mutant in vivo suggests a role for the heat shock regulatory network in *Campylobacter* gut colonization. In contrast, the *hrcA* mutant was not significantly affected in its in vitro growth neither in its ability to colonize the ileal loop. While the function of *hspR* and *hrcA* in *C. jejuni* is essentially unknown, the products of these genes were recently demonstrated to repress the transcription of the major heat shock proteins in *H. pylori* (*groESL*, *hrcA*, *grpE*, and *dnaK*) (38, 39). In addition, the HrcA-mediated repression was shown to be dependent on the binding of HspR to the promoter region (38). Interestingly, the transcription of the *cbpA-hspR-orf* operon was found to be exclusively regulated by HspR (38). Consequently, it is tempting to propose that the loss of HspR induces an increase in the abundance of heat shock proteins. Given that heat shock proteins are major immuno-dominant antigens, the over-expression of these proteins would likely contribute to host resistance (52) . As a result, the *hspR* mutants should be less capable of colonization and survival in the host. In addition, given the absence of colonization defect for the *hrcA* mutant, the amount of these proteins would not be increased at a sufficient level in this mutant to induce host resistance. In support of this hypothesis, *H. pylori hspR* and *hspR-hrcA* double mutants

were reported to have attenuated colonization efficiency in wild-type mice, while they were unaffected in IL-12-deficient mice (15). Furthermore, *C. jejuni* infected patients have been shown to develop humoral response against the heat shock protein DnaK (44).

In *E. coli*, *spoT* codes for a bi-functional enzyme able to catalyze the biosynthesis and the degradation of hyperphosphorylated guanine (p)ppGpp (1). In most eubacteria, (p)ppGpp has been shown to accumulate in response to stringent conditions, such as amino acid starvation, triggering the down-regulation of genes encoding the transcription and translation apparatus (1). As shown in Figure 6, the *spoT* mutant exhibits a competitive index of 9.10^{-6} in vivo. In contrast to this in vivo result, the in vitro competitive index was only 0.06, suggesting a significant in vivo specific defect. The decreased ability of the *C. jejuni spoT* mutant to colonize the host gut suggests an important role for the stringent response in vivo, likely allowing *Campylobacter* to deal with periods of nutrient starvation or other environmental stresses in the intestinal tract.

While the function of Cj0178 has not been characterized, this protein exhibits high homology with ferric-siderophore outer-membrane receptors. In addition, the expression of Cj0178 is Fur regulated and induced in response to iron starvation (26). Consequently, Cj0178 is probably required for the acquisition of iron from an uncharacterized siderophore. The Cj0178 mutant exhibits a competitive index of 0.05 in vivo and 0.37 in vitro. Given that *C. jejuni* NCTC 11168 does not seem to produce any siderophore (48), the growth defect of this mutant in vitro is unclear. The significant attenuation of the Cj0178 mutant in vivo is in agreement with its over-expression in our microarray experiment and might suggest an important role for this iron acquisition system in gut colonization.

Interestingly, while the *fliD* has a significant growth defect in vitro (exhibiting a a competitive index of 0.1), it colonizes the ileal loop as well as the wild-type strain (exhibiting a competitive index of 1.3). The *fliD* gene encodes a putative flagellar hook-associated protein. In *H. pylori*, *fliD* is an essential component in the assembly of a functional flagellum and is required for colonization of the gastric mucosa of mice (17). Similarly to *H. pylori*, the *C. jejuni fliD* mutant is non-motile and aflagellated (12). While the survival of the *fliD* mutant in the gut is in disagreement with the essential role of the flagellum in the colonization of the gastrointestinal tract, it is consistent with the characteristics of the RIL animal model. Indeed, the physical ligation of the rabbit intestinal tract likely favors the survival of mutants affected in their ability to adhere to the mucus or the intestinal epithelial cells. In addition, considering that flagellin is the major immunodominant antigen during infection (24), the loss of the flagellum structure should promote evasion of the immune system. Consequently, a *fliD* mutant will have an advantage over the wild-type in vivo and thus compete better during in vivo than in vitro growth.

Concluding remarks.

This genome-wide expression profiling study revealed important elements of the *Campylobacter* lifestyle during host intestinal tract colonization. In addition to the genes discussed above that have known or potential functions, many other genes of unknown function were also found to be differentially expressed between in vivo and in vitro growth, and therefore constitute many new directions for future investigations. The transcriptome pattern of *C. jejuni* in vivo was consistent with that expected in an

environment that is oxygen-limited, hyper-osmotic, nutrient restricted, and containing reactive oxygen compounds. Interestingly, the comparison of the *C. jejuni* transcriptomes between different rabbits revealed gene expression variability during the course of an infection. This flexibility in gene expression is probably essential for *Campylobacter* to adapt to the changing environment of the gut. Furthermore, the genes encoding proteins involved in flagellum biogenesis were found to be differentially expressed between rabbits. They were up-regulated in one rabbit and down-regulated (at a different level) in all others. While the up-regulation of these genes is in agreement with the role of the flagellum in gut colonization, the decreased expression of these genes probably reflects a bacterial strategy to evade the host response. Finally, regulation of both the heat shock response and the stringent response were found to be necessary for efficient colonization of the host gastrointestinal tract.

Acknowledgements.

The project described was supported by NIH grant numbers AI055612 and RR15564. The authors are grateful to all the staff from OU (Oklahoma University) and OSU microarray core facilities. The authors thank I. Turcot for providing helpful comments on the manuscript.

References

1. **Barker, M. M., T. Gaal, C. A. Josaitis, and R. L. Gourse.** 2001. Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro. J Mol Biol **305:**673-88.

2. **Boyce, J. D., I. Wilkie, M. Harper, M. L. Paustian, V. Kapur, and B. Adler.** 2002. Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. Infect Immun **70:**6871-9.

3. **Caldwell, M. B., R. I. Walker, S. D. Stewart, and J. E. Rogers.** 1983. Simple adult rabbit model for *Campylobacter jejuni* enteritis. Infect. Immun. **42:**1176-1182.

4. **Conway, T., and G. K. Schoolnik.** 2003. Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. Mol Microbiol **47:**879-89.

5. **Cotter, P. A., S. B. Melville, J. A. Albrecht, and R. P. Gunsalus.** 1997. Aerobic regulation of cytochrome d oxidase (*cydAB*) operon expression in *Escherichia coli*: roles of Fnr and ArcA in repression and activation. Mol Microbiol **25:**605-15.

6. **Crushell, E., S. Harty, F. Sharif, and B. Bourke.** 2004. Enteric *campylobacter*: purging its secrets? Pediatr Res **55:**3-12.

7. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol Microbiol **47:**103-18.

8. **Everest, P. H., H. Goossens, P. Sibbons, D. R. Lloyd, S. Knutton, R. Leece, J. M. Ketley, and P. H. Williams.** 1993. Pathological changes in the rabbit ileal loop model caused by *Campylobacter jejuni* from human colitis. J Med Microbiol **38:**316-21.

9. **Fox, J. G., A. B. Rogers, M. T. Whary, Z. Ge, N. S. Taylor, S. Xu, B. H. Horwitz, and S. E. Erdman.** 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun **72:**1116-25.

10. **Fuller, T. E., M. J. Kennedy, and D. E. Lowery.** 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. Microb Pathog **29:**25-38.

11. **Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell.** 2004. The Genome-Sequenced Variant of *Campylobacter jejuni* NCTC 11168 and the Original Clonal Clinical Isolate Differ Markedly in Colonization, Gene Expression, and Virulence-Associated Phenotypes. J Bacteriol **186:**503-517.

12. **Golden, N. J., and D. W. Acheson.** 2002. Identification of motility and autoagglutination *Campylobacter jejuni* mutants by random transposon mutagenesis. Infect Immun **70:**1761-71.

13. **Guiney, D. G.** 1997. Regulation of bacterial virulence gene expression by the host environment. J. Clin. Invest. **99:**565-9.

14. **Harris, A. G., J. E. Wilson, S. J. Danon, M. F. Dixon, K. Donegan, and S. L. Hazell.** 2003. Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. Microbiology **149:**665-72.

15. **Hoffman, P. S., N. Vats, D. Hutchison, J. Butler, K. Chisholm, G. Sisson, A. Raudonikiene, J. S. Marshall, and S. J. Veldhuyzen van Zanten.** 2003. Development of an interleukin-12-deficient mouse model that is permissive for colonization by a motile KE26695 strain of *Helicobacter pylori*. Infect Immun **71:**2534-41.

16. **Karlyshev, A. V., M. J. Pallen, and B. W. Wren.** 2000. Single-primer PCR procedure for rapid identification of transposon insertion sites. Biotechniques **28:**1078, 1080, 1082.

17. **Kim, J. S., J. H. Chang, S. I. Chung, and J. S. Yum.** 1999. Molecular cloning and characterization of the *Helicobacter pylori fliD* gene, an essential factor in flagellar structure and motility. J Bacteriol **181:**6969-76.

18. **Lin, J., L. O. Michel, and Q. Zhang.** 2002. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. Antimicrob Agents Chemother **46:**2124-31.

19. **Lin, J., O. Sahin, L. O. Michel, and Q. Zhang.** 2003. Critical Role of Multidrug Efflux Pump CmeABC in Bile Resistance and In Vivo Colonization of *Campylobacter jejuni*. Infect Immun **71:**4250-9.

20. **McSweegan, E., and R. I. Walker.** 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect Immun **53:**141-8.

21. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607-625.

22. **Merrell, D. S., D. L. Hava, and A. Camilli.** 2002. Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. Mol. Microbiol. **43:**1471-91.

23. **Monteville, M. R., J. E. Yoon, and M. E. Konkel.** 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. Microbiology **149:**153-65.

24. **Nachamkin, I., and A. M. Hart.** 1985. Western blot analysis of the human antibody response to *Campylobacter jejuni* cellular antigens during gastrointestinal infection. J Clin Microbiol **21:**33-8.

25. **Newell, D. G., H. McBride, and J. M. Dolby.** 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. J Hyg (Lond) **95:217-27.**

26. Palyada, K., D. Threadgill, and A. Stintzi. 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol **186:**4714-29.

27. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M.-A. Rajandream, K. M. Rutherford, A. H. M. V. Vliet, S. Whitehead, and B. G. Barell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-668.

28. **Pavlovskis, O. R., D. M. Rollins, R. L. Harberberger, A. E. Green, L. Habash, S. Stroko, and R. I. Walker.** 1991. Significance of flagella in colonization resistance of rabbits immunized with *Campylobacter* spp. Infect. Immun. **66:**938-943.

29. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X. Z. Huang, D. J. Kopecko, A. L. Bourgeois, J. L. Fauchere, and M. J. Blaser.** 1998. Mutation in the *peb1A* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect Immun **66:**938-43.

30. **Poly, F., D. Threadgill, and A. Stintzi.** 2004. Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. J Bacteriol **186:**4781-95.

31. **Purdy, D., S. Cawthraw, J. H. Dickinson, D. G. Newell, and S. F. Park.** 1999. Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonization. Appl Environ Microbiol **65:**2540-6.

32. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol **54:**881-941.

33. **Richardson, D. J.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146 (Pt 3):**551-71.

34. **Romeo, T.** 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol Microbiol **29:**1321-30.

35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

36. **Sellars, M. J., S. J. Hall, and D. J. Kelly.** 2002. Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. J Bacteriol **184:**4187-96.

37. **Sorensen, M. A., J. Fricke, and S. Pedersen.** 1998. Ribosomal protein S1 is required for translation of most, if not all, natural mRNAs in *Escherichia coli* in vivo. J Mol Biol **280:**561-9.

38. **Spohn, G., A. Danielli, D. Roncarati, I. Delany, R. Rappuoli, and V. Scarlato.** 2004. Dual control of *Helicobacter pylori* heat shock gene transcription by HspR and HrcA. J Bacteriol **186:**2956-65.

39. **Spohn, G., I. Delany, R. Rappuoli, and V. Scarlato.** 2002. Characterization of the HspR-mediated stress response in *Helicobacter pylori*. J Bacteriol **184:**2925-30.

40. **Stintzi, A.** 2003. Gene Expression Profile of *Campylobacter jejuni* in Response to Growth Temperature Variation. J Bacteriol **185:**2009-16.

41. **Stintzi, A., and L. Whitworth.** 2003. Investigation of the *Campylobacter jejuni* cold-shock response by global transcript profiling. Genome Letters **2:**18-27.

42. **Szymanski, C. M., D. H. Burr, and P. Guerry.** 2002. *Campylobacter* protein glycosylation affects host cell interactions. Infect Immun **70:**2242-4.

43. **Talaat, A. M., P. Hunter, and S. A. Johnston.** 2000. Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis. Nat Biotechnol **18:**679-82.

44. **Thies, F. L., H. Karch, H. P. Hartung, and G. Giegerich.** 1999. Cloning and expression of the *dnaK* gene of *Campylobacter jejuni* and antigenicity of heat shock protein 70. Infect Immun **67:**1194-200.

45. **Thies, F. L., H. Karch, H. P. Hartung, and G. Giegerich.** 1999. The ClpB protein from *Campylobacter jejuni*: molecular characterization of the encoding gene and antigenicity of the recombinant protein. Gene **230:**61-7.

46. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A **98:**5116-21.

47. **van Spreeuwel, J. P., G. C. Duursma, C. J. Meijer, R. Bax, P. C. Rosekrans, and J. Lindeman.** 1985. *Campylobacter coli*tis: histological immunohistochemical and ultrastructural findings. Gut **26:**945-51.

48. **van Vliet, A. H., J. M. Ketley, S. F. Park, and C. W. Penn.** 2002. The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. FEMS Microbiol Rev **26:**173-86.

49. **van Vliet, A. H. M., and J. M. Ketley.** 2001. Pathogenesis of *Campylobacter* infection. J Applied Microb. **90:**45S-56S.

50. **Vijaranakul, U., M. J. Nadakavukaren, B. L. de Jonge, B. J. Wilkinson, and R. K. Jayaswal.** 1995. Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. J Bacteriol **177:**5116-21.

51. **Wosten, M. M., J. A. Wagenaar, and J. P. Van Putten.** 2004. The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. J Biol Chem.

52. **Wu, Y. L., L. H. Lee, D. M. Rollins, and W. M. Ching.** 1994. Heat shock- and alkaline pH-induced proteins of *Campylobacter jejuni*: characterization and immunological properties. Infect. Immun. **62:**4256-60.

53. **Wurmbach, E., T. Yuen, B. J. Ebersole, and S. C. Sealfon.** 2001. Gonadotropin-releasing hormone receptor-coupled gene network organization. J. Biol. Chem. **276:**47195-201.

54. **Xu, Q., M. Dziejman, and J. J. Mekalanos.** 2003. Determination of the transcriptome of *Vibrio cholerae* during intraintestinal growth and midexponential phase in vitro. Proc Natl Acad Sci U S A **100:**1286-91.

55. **Yao, R., R. A. Alm, T. J. Trust, and P. Guerry.** 1993. Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. Gene **130:**127-30.

56. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. Mol Microbiol **14:**883-93.

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	*endA1 hsdR17* ($r_k^-m_k^-$) *supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYAargF U169deoR [f80dlac*Δ*(lacZ0 M15]*	Invitrogen
C. jejuni		
NCTC11168	*C. jejuni* NCTC 11168	NCTC
AS 283	NCTC11168ΔCj0571	This study
AS 287	NCTC11168Δ*spoT*	This study
AS272	NCTC11168Δ*hspR*	This study
AS277	NCTC11168Δ*hrcA*	This study
AS211	NCTC11168ΔCj0178	(26)
AS317	NCTC11168ΔCj0341	This study
AS318	NCTC11168Δ*fliD*	This study
Plasmids		
pUC19	Cloning and suicide vector, Amp^r	Biolabs
pRY111	*cam* resistance gene	(55)
pAS282	pUC19 carrying ΔCj0571*::cam*	This study
pAS271	pUC19 carrying Δ*hspR::cam*	This study
pAS315	pUC19 carrying Δ*hrcA::cam*	This study
pAS286	pUC19 carrying Δ*spoT::cam*	This study

[a]cam, chloramphenicol resistance gene;
Amp^r, ampicillin resistant

Table 2: Primers used in this study.

Primer	DNA sequence from 5' to 3' (restriction site)[a]
Primers used for gene cloning	
spoT-01	ATGC**GAATTC**GAGATTTTAACCTATCCTTGACAC (*Eco*RI)
spoT-02	ATGC**CTGCAG**CCATGATGCCATTCTTGAAA (*Pst*I)
Cj0571-01	ATGC**GAATTC**ATGCAAGAAAATTTCATACGC (*Eco*RI)
Cj0571-02	ATGC**CTGCAG**TCCCGTTGTAGCATCTTTTG (*Pst*I)
hrcA-01	ACGT**GGTACC**AATAGAGTGCTAGATATGAAGGAA (*Kpn*I)
hrcA-02	ACGT**GGTACC**GATGGTTTTCACTATCTACATGAA (*Kpn*I)
hspR-01	ACGT**GGTACC**GGAAAACAAAGCAGAAATGGAGC (*Kpn*I)
hspR-02	ACGT**GGTACC**GCACCACTGCGGTTGAAGAAA (*Kpn*I)
Primers used for inverse PCR	
spoT-03	ATGC**GGATCC**AAAACAGGAAAAGCAAAAGCA (*Bam*HI)
spoT-04	ATGC**GGATCC**AATGCCTTTTCTTTGCAT (*Bam*HI)
Cj0571-03	ATGC**GGATCC**GCTTAATTTTCCCAAAGCAAA (*Bam*HI)
Cj0571-04	ATGC**GGATCC**AGAACTGAAAATACGGCTAGAAGA (*Bam*HI)
hrcA-03	ACGT**CCATGG**ATTGCTTCTATAGCAAATCAA (*Nco*I)
hrcA-04	ACGT**GCGGCCGC**AAGCTCTTAGATTGTCAAATTCAC (*Not*I)
hspR-03	ACGT**CCATGG**TTGCCATCGGTTCTACTTGG (*Nco*I)
hspR-04	ACGT**GCGGCCGC**GAATCAATCTTGCTGGAGTA (*Not*I)

[a]The restriction sites used for cloning are highlighted in bold and indicated in parenthesis.

Figure 1

Detection of *C. jejuni* transcriptome in vivo. Panels A and B show the rabbit ileal loops 48 hours post-inoculation with *C. jejuni* or PBS buffer, respectively. The arrows indicate intestine distended with gas and fluid accumulation. Total RNA was extracted from the intestinal contents, reverse transcribed using *C. jejuni* specific 3' primer, and labeled with the Cy5 dye. This labeled cDNA was co-hybridized to the microarray with Cy3 labeled cDNA, obtained from in vitro grown bacterial RNA.

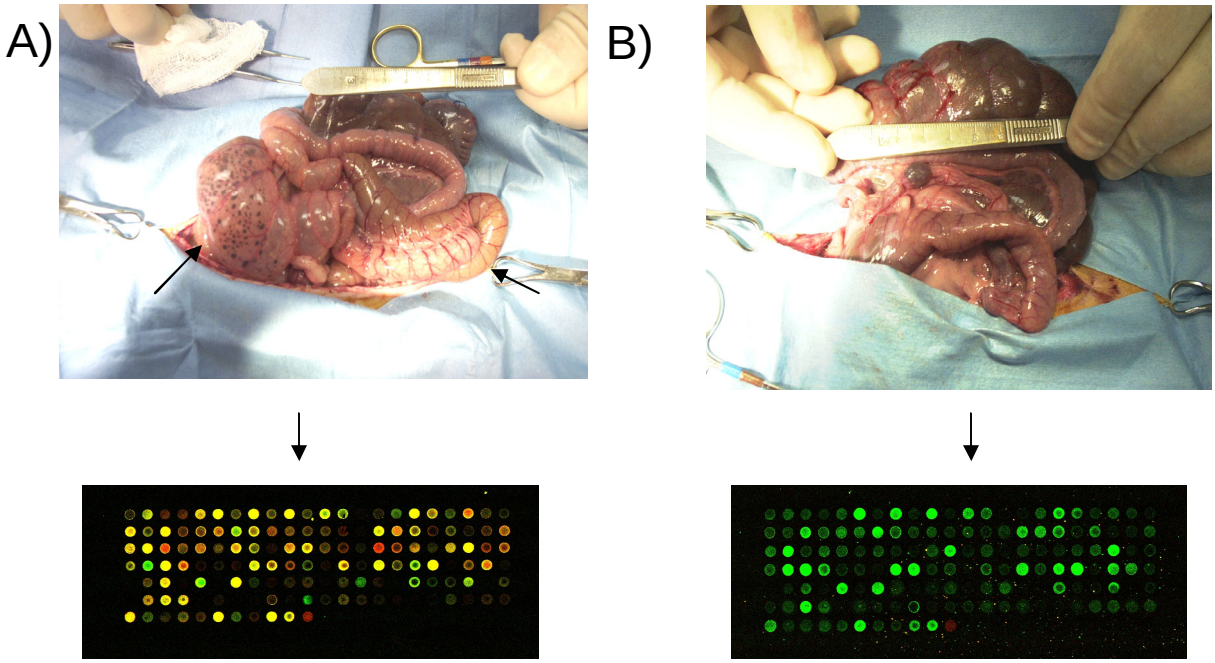
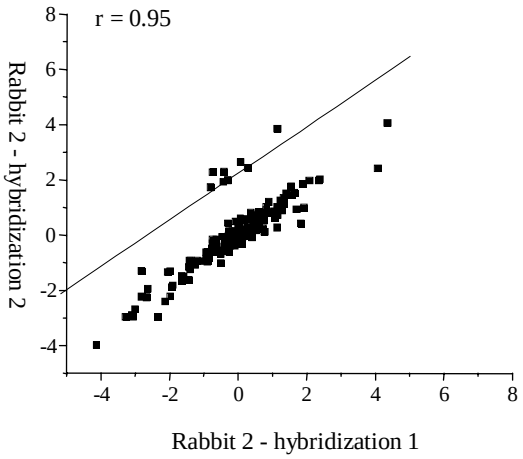

A)
B)

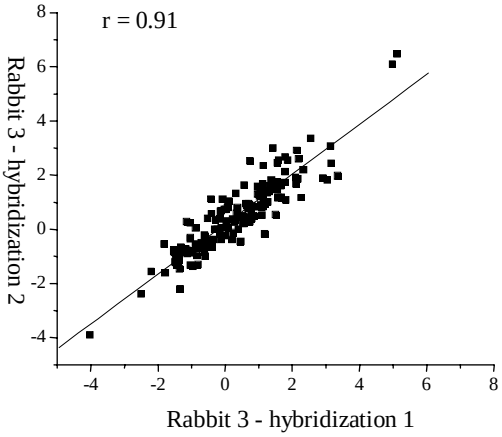
Figure 2

Scatter plots showing the relationship between the $\log_2$ value of the gene expression ratio obtained from hybridization experiments with bacterial cDNA derived from the same rabbit (Panels A and B) or from two different rabbits (Panel C). The solid lines represent the linear regression fit of the data.

A)


r = 0.95
Rabbit 2 - hybridization 2
Rabbit 2 - hybridization 1

B)


r = 0.91
Rabbit 3 - hybridization 2
Rabbit 3 - hybridization 1

C)

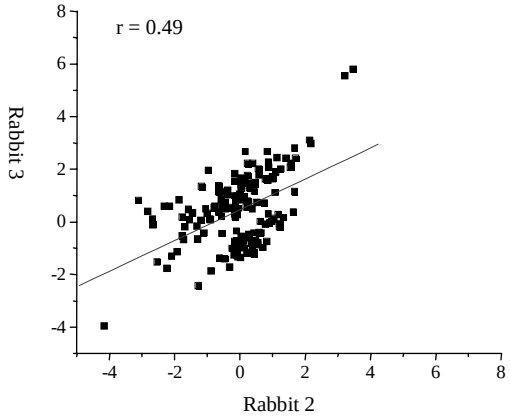

r = 0.49
Rabbit 3
Rabbit 2

Figure 3

Global view of genes with similar expression pattern between rabbits grouped by functional categories according to the Sanger Center *C. jejuni* genome database. Each row represents one gene and each column represents the expression profile in one rabbit (the mean of the fold change expression ratio of the technical replicates). The column label corresponds to the rabbit numbering. An increasing red intensity denotes genes that have their expression significantly increased in vivo compared to in vitro growth and an increasing green intensity indicates genes that have their expression significantly decreased in vivo compared to in vitro growth. A gray color indicates missing data. Genes with unknown functions are not represented.

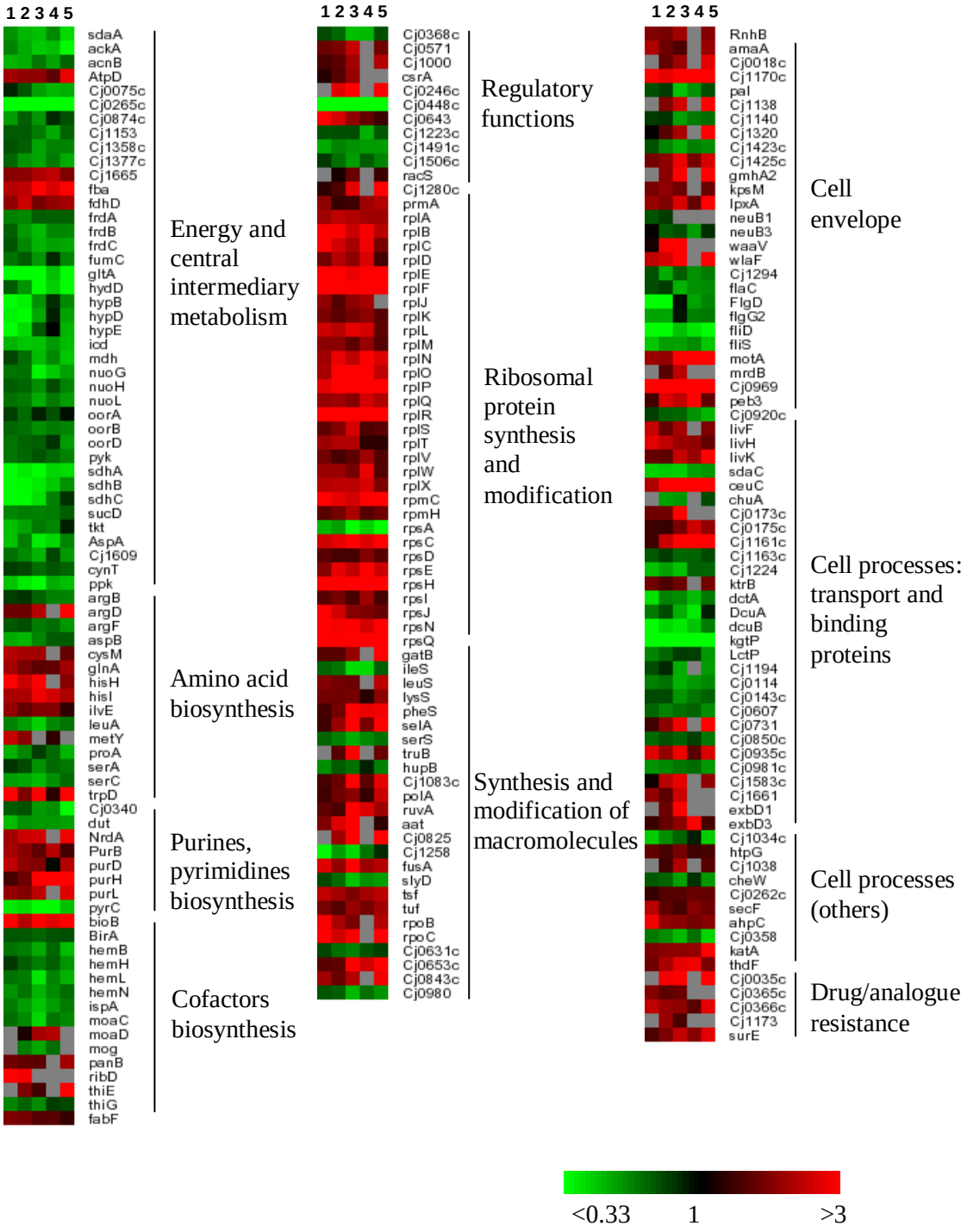

1 2 3 4 5
sdaA
ackA
acnB
AtpD
Cj0075c
Cj0265c
Cj0874c
Cj1153
Cj1358c
Cj1377c
Cj1665
fba
fdhD
frdA
frdB
frdC
fumC
gltA
hydD
hypB
hypD
hypE
icd
mdh
nuoG
nuoH
nuoL
oorA
oorB
oorD
pyk
sdhA
sdhB
sdhC
sucD
tkt
AspA
Cj1609
cynT
ppk
argB
argD
argF
aspB
cysM
glnA
hisH
hisI
ilvE
leuA
metY
proA
serA
serC
trpD
Cj0340
dut
NrdA
PurB
purD
purH
purL
pyrC
bioB
BirA
hemB
hemH
hemL
hemN
ispA
moaC
moaD
mog
panB
ribD
thiE
thiG
fabF
Energy and central intermediary metabolism
Amino acid biosynthesis
Purines, pyrimidines biosynthesis
Cofactors biosynthesis
1 2 3 4 5
Cj0368c
Cj0571
Cj1000
csrA
Cj0246c
Cj0448c
Cj0643
Cj1223c
Cj1491c
Cj1506c
racS
Cj1280c
prmA
rplA
rplB
rplC
rplD
rplE
rplF
rplJ
rplK
rplL
rplM
rplN
rplO
rplP
rplQ
rplR
rplS
rplT
rplV
rplW
rplX
rpmC
rpmH
rpsA
rpsC
rpsD
rpsE
rpsH
rpsI
rpsJ
rpsN
rpsQ
gatB
ileS
leuS
lysS
pheS
selA
serS
truB
hupB
Cj1083c
polA
ruvA
aat
Cj0825
Cj1258
fusA
slyD
tsf
tuf
rpoB
rpoC
Cj0631c
Cj0653c
Cj0843c
Cj0980
Regulatory functions
Ribosomal protein synthesis and modification
Synthesis and modification of macromolecules
1 2 3 4 5
RnhB
amaA
Cj0018c
Cj1170c
pal
Cj1138
Cj1140
Cj1320
Cj1423c
Cj1425c
gmhA2
kpsM
lpxA
neuB1
neuB3
waaV
wlaF
Cj1294
flaC
FlgD
flgG2
fliD
fliS
motA
mrdB
Cj0969
peb3
Cj0920c
livF
livH
livK
sdaC
ceuC
chuA
Cj0173c
Cj0175c
Cj1161c
Cj1163c
Cj1224
ktrB
dctA
DcuA
dcuB
kgtP
LctP
Cj1194
Cj0114
Cj0143c
Cj0607
Cj0731
Cj0850c
Cj0935c
Cj0981c
Cj1583c
Cj1661
exbD1
exbD3
Cj1034c
htpG
Cj1038
cheW
Cj0262c
secF
ahpC
Cj0358
katA
thdF
Cj0035c
Cj0365c
Cj0366c
Cj1173
surE
Cell envelope
Cell processes: transport and binding proteins
Cell processes (others)
Drug/analogue resistance
<0.33
1
>3

Figure 4

Global view of genes with a variable expression pattern between rabbits. Each row represents one gene. Columns 1, 2, 3, 4, and 5 represent the expression profile in rabbit 1, 2, 3, 4, and 5 respectively. For each rabbit, the microarray data correspond to the mean of the fold change expression ratio of the technical replicates. Red and green denotes transcripts that have their abundance increased or decreased in vivo compared to in vitro growth, respectively. The red and green intensities are proportional to the fold increase or decrease with maximal fold changes in transcript abundance of 3 and 0.33, respectively. A gray color denotes missing data.

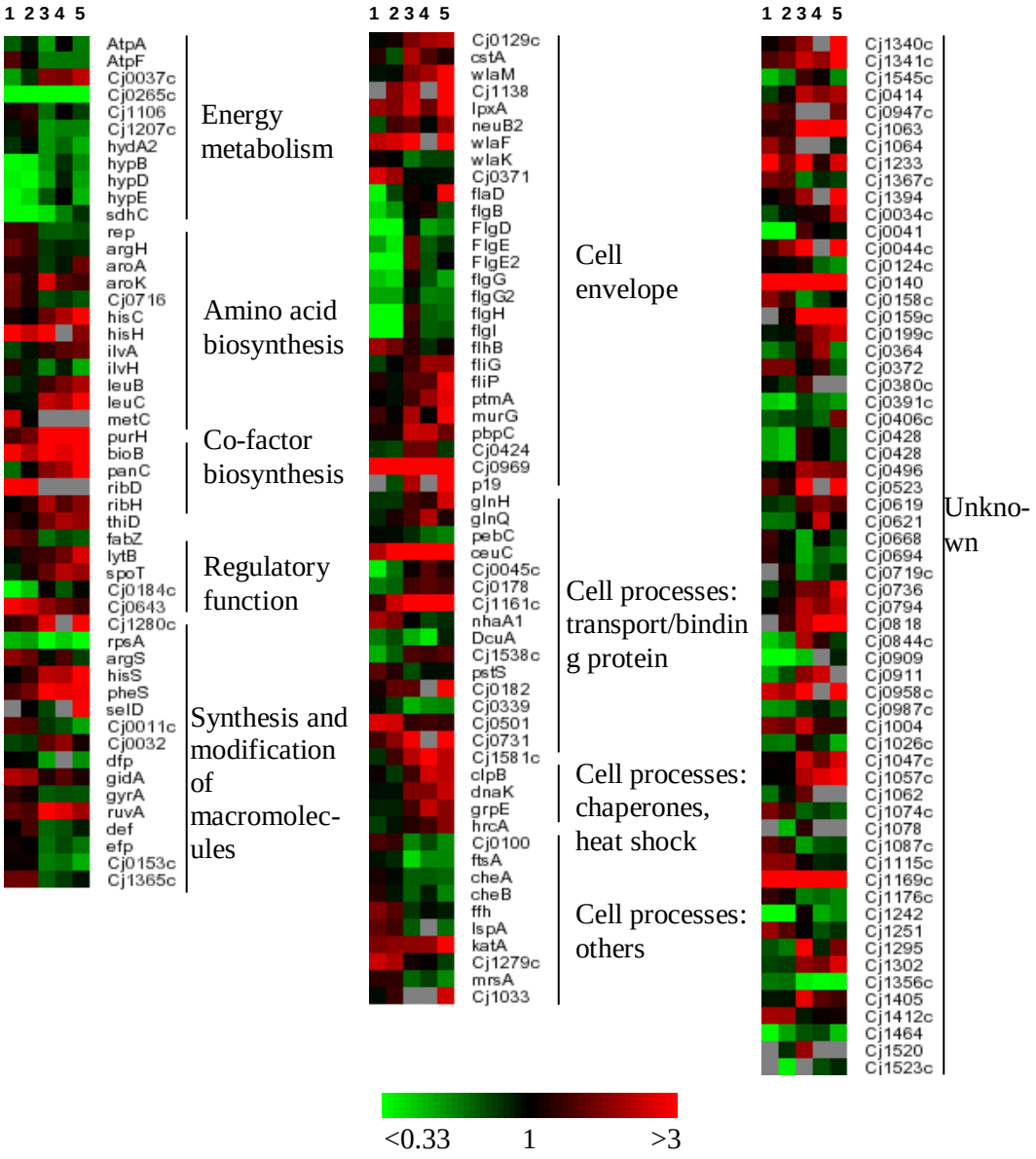

1 2 3 4 5
AtpA
AtpF
Cj0037c
Cj0265c
Cj1106
Cj1207c
hydA2
hypB
hypD
hypE
sdhC
Energy metabolism
rep
argH
aroA
aroK
Cj0716
hisC
hisH
ilvA
ilvH
leuB
leuC
metC
Amino acid biosynthesis
purH
bioB
panC
ribD
ribH
thiD
fabZ
Co-factor biosynthesis
lytB
spoT
Cj0184c
Cj0643
Regulatory function
Cj1280c
rpsA
argS
hisS
pheS
selD
Cj0011c
Cj0032
dfp
gidA
gyrA
ruvA
def
efp
Cj0153c
Cj1365c
Synthesis and modification of macromolec-ules
1 2 3 4 5
Cj0129c
cstA
wlaM
Cj1138
lpxA
neuB2
wlaF
wlaK
Cj0371
flaD
flgB
FlgD
FlgE
FlgE2
flgG
flgG2
flgH
flgI
flhB
fliG
fliP
ptmA
murG
pbpC
Cj0424
Cj0969
Cell envelope
p19
glnH
glnQ
pebC
ceuC
Cj0045c
Cj0178
Cj1161c
nhaA1
DcuA
Cj1538c
pstS
Cj0182
Cj0339
Cj0501
Cj0731
Cell processes: transport/bindin g protein
Cj1581c
clpB
dnaK
grpE
hrcA
Cell processes: chaperones, heat shock
Cj0100
ftsA
cheA
cheB
ffh
lspA
katA
Cj1279c
mrsA
Cj1033
Cell processes: others
1 2 3 4 5
Cj1340c
Cj1341c
Cj1545c
Cj0414
Cj0947c
Cj1063
Cj1064
Cj1233
Cj1367c
Cj1394
Cj0034c
Cj0041
Cj0044c
Cj0124c
Cj0140
Cj0158c
Cj0159c
Cj0199c
Cj0364
Cj0372
Cj0380c
Cj0391c
Cj0406c
Cj0428
Cj0428
Cj0496
Cj0523
Cj0619
Cj0621
Cj0668
Cj0694
Cj0719c
Cj0736
Cj0794
Cj0818
Cj0844c
Cj0909
Cj0911
Cj0958c
Cj0987c
Cj1004
Cj1026c
Cj1047c
Cj1057c
Cj1062
Cj1074c
Cj1078
Cj1087c
Cj1115c
Cj1169c
Cj1176c
Cj1242
Cj1251
Cj1295
Cj1302
Cj1356c
Cj1405
Cj1412c
Cj1464
Cj1520
Cj1523c
Unkno-wn
<0.33
1
>3

Figure 5

Competitive colonization ability of 7 mutants (*hrcA*, Cj0178, Cj0571, *spoT*, Cj0341, *hspR*, and *fliD*). The strains were pooled with the parent strain *C. jejuni* NCTCCC 11168 (constituting the input pool) and inoculated into 4 rabbit ileal loops. Forty-eight hours post-inoculation, the intestinal content was recovered and processed for chromosomal DNA extraction. The number of bacteria was estimated by quantitative real-time PCR for each mutant as described in the Materials and Methods section. The normalized competitive ratio corresponds to the ratio of the number of mutant cells to the total number of bacterial cells in the input pool divided by the ratio of the number of mutants to the total number of bacteria in the recovered pool. The data is the mean of eight determinations (four biological replicates with two technical replicates each), and the error bars represent the standard deviations.

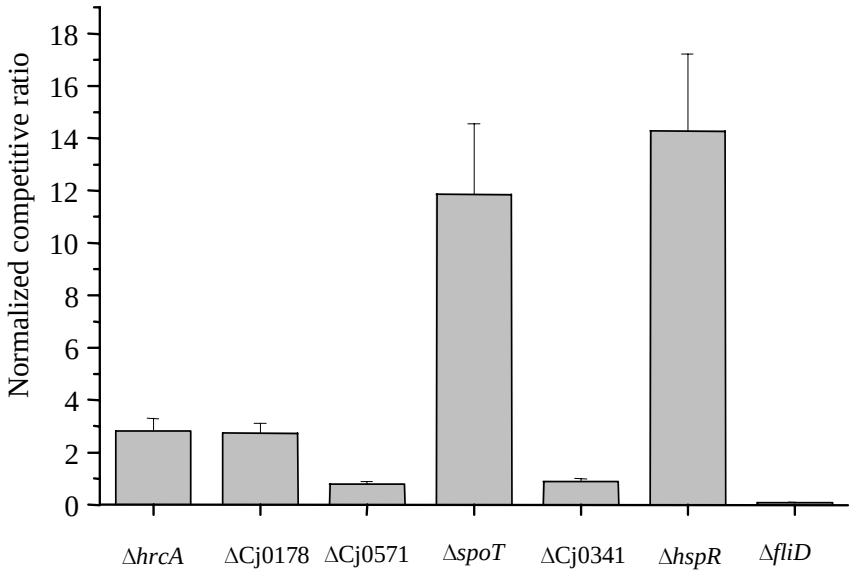
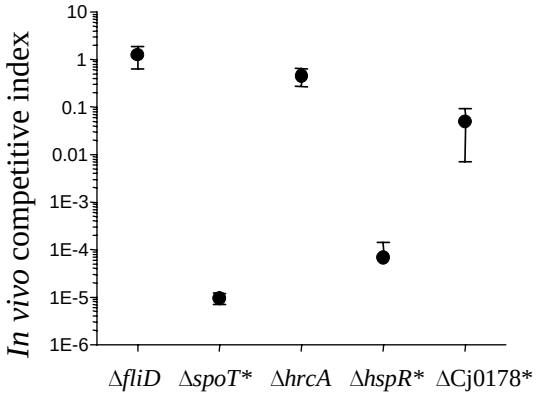

Normalized competitive ratio
0
2
4
6
8
10
12
14
16
18
ΔhrcA
ΔCj0178
ΔCj0571
ΔspoT
ΔCj0341
ΔhspR
ΔfliD

Figure 6

In vivo (panel A) and in vitro (panel B) competition assays. The in vivo competitive index is the ratio of the mutant to the wild-type strain recovered in the ileal loop 48 hours post-infection. Four loops were infected with a mixture of each mutant and the wild-type strain at a ratio of 1 to 1. The in vitro competitive index is the ratio of the mutant to the wild-type strain in MH broth at late log phase. The in vitro competition assay was performed in triplicate. The error bars indicate the standard deviations. The symbol * indicates a statistical significance (*P* value <0.001).

A)


In vivo competitive index
10
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
ΔfliD
ΔspoT*
ΔhrcA
ΔhspR*
ΔCj0178*

B)

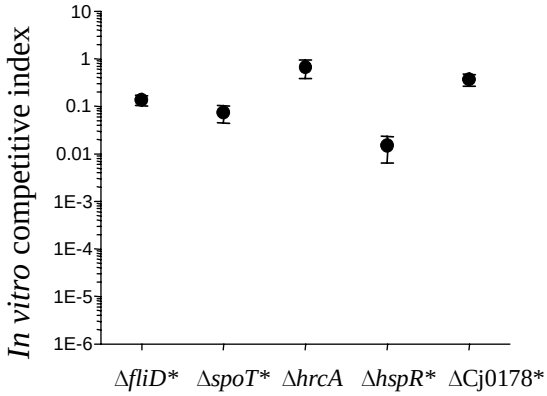
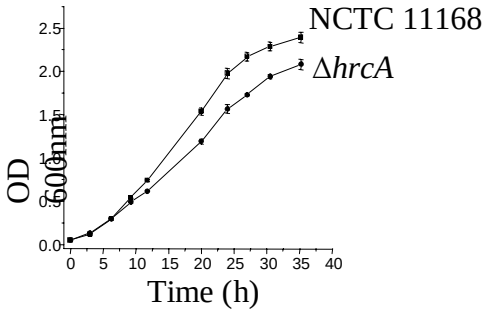

In vitro competitive index
10
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
ΔfliD*
ΔspoT*
ΔhrcA
ΔhspR*
ΔCj0178*

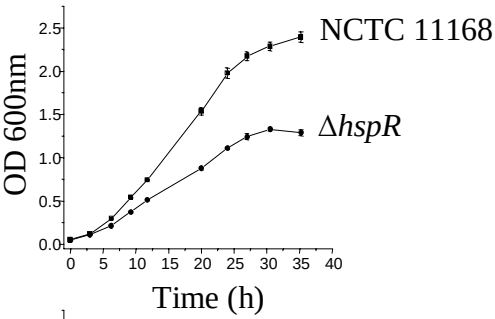
Figure 7

Growth kinetics of *C. jejuni* NCTC 11168 and 5 mutants, Δ*hrcA* (panel A), Δ*hspR* (panel B), Δ*spoT* (panel C), ΔCj0178 (panel D), and Δ*fliD* (panel E). Biphasic MH cultures were incubated at 37°C under microaerophilic conditions. The growth kinetics were performed in triplicate and the error bars represent the standard deviations.

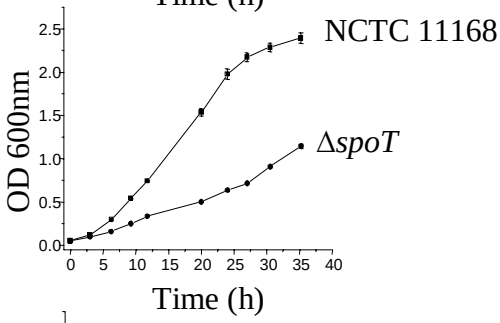
A)


NCTC 11168
Δ*hrcA*
OD 600nm
Time (h)

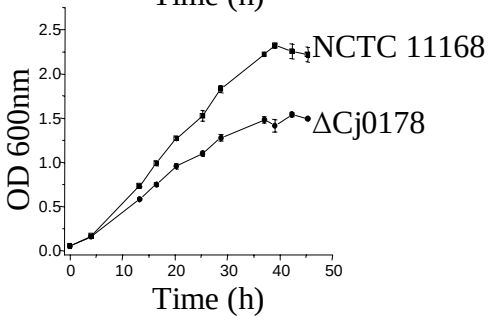
B)


NCTC 11168
Δ*hspR*
OD 600nm
Time (h)

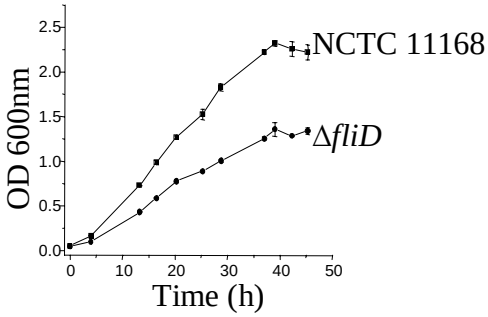
C)


NCTC 11168
Δ*spoT*
OD 600nm
Time (h)

D)


NCTC 11168
ΔCj0178
OD 600nm
Time (h)

E)


NCTC 11168
Δ*fliD*
OD 600nm
Time (h)

VITA

Hemant Kashinath Naikare

Candidate for the Degree of
Doctor of Philosophy

THESIS: FERROUS AND FERRIC IRON ACQUSITION IN *CAMPYLOBACTER JEJUNI*

Major Field: Veterinary Biomedical Sciences

Education:

Degree	Discipline	University	Year
Master of Veterinary Sciences in Veterinary Microbiology (MVSc-Micro)	Veterinary Microbiology	Konkan Krishi Vidyapeeth, India	2000
Bachelor of Veterinary sciences (BVSc & AH)	Veterinary Sciences & Animal Husbandry	Konkan Krishi Vidyapeeth, India	1998

Awards / Honors:

Research: Graduate Research Assistant in Veterinary Biomedical Sciences (2001-present)

Teaching: Graduate Teaching Assistant for Veterinary Microbiology (2000-2001)

University Merit Scholarship for BVSc & AH (1996-1997)

Affiliations:

Member-American Society for Microbiology (ASM) since 2002

Name: Hemant K. Naikare Date of Degree: May, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: FERROUS AND FERRIC IRON ACQUISITION IN *CAMPYLOBACTER JEJUNI*

Pages in Study: 276 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences

Scope and Method of study: Identification of colonization and virulence determinants that contribute to *C. jejuni* infection and survival in the gastrointestinal tract of the animal host would be an important tool in designing intervention strategies to combat the infection. Availability of iron in the host is a key environmental factor for controlling the establishment of many bacterial infections. The global aim of this project was to decipher the molecular mechanisms of iron acquisition in *C. jejuni*. We report the characterization of FeoB mediated ferrous iron and TonB mediated ferric iron acquisition in *C. jejuni*. We used directed mutagenesis to functionally disrupt the *feoB* and *tonB* genes. By performing a series of *in vitro* experiments such as $^{55}Fe^{2+}$ uptake assays, cell culture based assays and microarrays; we have studied the role of FeoB in iron transport, iron accumulation and intracellular survival within human and porcine intestinal epithelial cells. TonB systems were characterized *in vitro* using growth promotion assays, ferric-enterobactin assays and cell culture. The FeoB and TonB systems were extensively assessed for their role in the *in vivo* colonization of the gastrointestinal tract of the chicken, rabbit ileal loop and the colostrums-deprived neonatal piglet models.

Findings and Conclusions: The *feoB* mutant was significantly impaired in iron uptake and was demonstrated to have decreased cellular iron accumulation. FeoB was found to play significant role in the intracellular survival of *C. jejuni* within human and porcine epithelial cells. The *feoB* mutant was significantly affected in its ability to colonize the gastrointestinal tract of chicks, rabbits and neonatal piglets thus highlighting its importance in the gut colonization. Growth promotion assays and ferric-enterobactin assays revealed that TonBs played specific and redundant roles in acquisition of iron from different sources. TonB3 was found to be specifically required for enterobactin utilization, whereas TonB2 and TonB3 were together required for the uptake of hemin and hemoglobin. All the *tonB* mutants were significantly affected in their ability to colonize the gastrointestinal tract of neonatal piglets. With the exception of *tonb2* mutant, all the other series of single and double *tonB* mutants were affected in their ability to colonize the chicken ceca. Thus, combining our *in vitro* and *in vivo* characterization of the FeoB and TonB systems, it is clear that both the systems contribute significantly in iron transport and gastrointestinal colonization of the animal models.

ADVISOR'S APPROVAL: _____Dr.Alain Stintzi_____