Major Role for FeoB in Campylobacter jejuni Ferrous Iron Acquisition, Gut Colonization, and Intracellular Survival

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To assess the importance of ferrous iron acquisition in Campylobacter physiology and pathogenesis, we disrupted and characterized the Fe²⁺ iron transporter, FeoB, in Campylobacter jejuni NCTC 11168, 81-176, and ATCC 43431. The feoB mutant was significantly affected in its ability to transport Fe²⁺. It accumulated half the amount of iron than the wild-type strain during growth in an iron-containing medium. The intracellular iron of the feoB mutant was localized in the periplasmic space versus the cytoplasm for the wild-type strain. These results indicate that the feoB gene of C. jejuni encodes a functional ferrous iron transport system. Reverse transcriptase PCR analysis revealed the cotranscription of feoB and Cj1397, which encodes a homolog of Escherichia coli feoA. C. jejuni 81-176 feoB mutants exhibited reduced ability to persist in human INT-407 embryonic intestinal cells and porcine IPEC-1 small intestinal epithelial cells compared to the wild type. C. jejuni NCTC 11168 feoB mutant was outcompeted by the wild type for colonization and/or survival in the rabbit ileal loop. The feoB mutants of the three C. jejuni strains were significantly affected in their ability to colonize the chick cecum. And finally, the three feoB mutants were outcompeted by their respective wild-type strains for infection of the intestinal tracts of colostrum-deprived piglets. Taken together, these results demonstrate that FeoB-mediated ferrous iron acquisition contributes significantly to colonization of the gastrointestinal tract during both commensal and infectious relationship, and thus it plays an important role in Campylobacter pathogenesis.

The ferrous iron transporter was isolated for the first time in Escherichia coli, more than 15 years ago, by Hantke (12). The two E. coli genes, feoA and feoB, were then fully characterized and sequenced by Kammler and coworkers 6 years later (14). E. coli mutants of both genes were shown to be significantly affected in their ability to transport ferrous iron (14). During the past 10 years, the ferrous iron acquisition system has been experimentally identified in seven additional microbes: Porphyromonas gingivalis (6), Leptospira biflexa (16), Helicobacter pylori (35), Shigella flexneri (27), Salmonella enterica serovar Typhimurium (4, 33), Legionella pneumophila (26), and the cyanobacterium Synechocystis sp. (15). Interestingly, the FeoB-mediated ferrous iron acquisition has been found to play an essential role in bacterial virulence (4, 6), intracellular survival (26, 27), replication in macrophage (4), and/or gastrointestinal tract colonization (32, 33, 35).

In E. coli, the feoA and feoB genes are organized in an operonic structure with a third gene named fecO (11). The genes feoA and fecO encode two small proteins of 75 and 78 amino acids, respectively. The function of these proteins in ferrous iron transport is still unknown. The fecO gene encodes an integral cytoplasmic membrane protein of 773 amino acids. FeoB homologs are found in all bacterial kingdoms from Chaeae to gram-positive and gram-negative bacteria (11). Interestingly, the FeoB protein shares homology with GTP-binding proteins. It contains four of the five GTPase signature motifs. Mutational and GTP binding studies have demonstrated the requirement of GTP/GDP binding for ferrous iron uptake (18). Although it is clear that FeoB is required for ferrous iron acquisition, its precise function in iron transport and whether or not FeoB binds iron is not known (11, 18).

Campylobacter jejuni is a major etiological agent of gastroenteritis worldwide and causes up to 2.5 million illnesses every year in the United States alone (19). This pathogen colonizes primarily the gastrointestinal tract of warm-blooded animals. The oxygen-reduced environment of the gut will likely favor the reduction of iron to its ferrous state. Consequently, ferrous iron might be an important iron source for enteric pathogens such as C. jejuni given that feoB mutants of E. coli, H. pylori, and S. enterica serovar Typhimurium, have been shown to be attenuated in their ability to colonize the host gastrointestinal tract (32, 33, 35). The analysis of the C. jejuni NCTC 11168 genome reveals that it carries an feoAB-like operon (21). The C. jejuni FeoB protein shares 50% identity and 69% similarity at the amino acid level with the FeoB protein of H. pylori. Surprisingly, and in contrast to all of the other studied and characterized FeoB proteins, the FeoB homolog in C. jejuni was recently found to not be required for ferrous iron uptake (25). This conclusion was based on the absence of ferrous iron uptake defect in the feoB mutant of two strains of C. jejuni, M129 and F38011 (25). The genomes of these two strains have not been sequenced. Consequently, it is unknown whether these strains harbor an additional FeoB homolog or an alternative ferrous transport system, which could explain the observed phenotype of the feoB mutants. Given that the feoB mutant of C. jejuni NCTC 11168 was not tested in the present

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TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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* Amp'r, ampicillin resistance gene.

study and that its genome does not contain any other FeoB homolog, we reevaluated and further studied the function of FeoB in ferrous iron acquisition. Here, we present the role of FeoB in C. jejuni iron acquisition, gut colonization, and intracellular survival.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in the present study are listed in Table 1. E. coli DH5α was 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 ampicillin (100 µg/ml) or kanamycin (30 µg/ml). C. jejuni strains were routinely grown at 37°C in a MACS-VA500 microaerophilic workstaton (Don Whitley, West Yorkshire, England) or in an atmosphere of 83% N2, 4% H2, 2% O2, and 5% CO2 on Mueller-Hinton (MH) agar plates, MH broth medium, MH broth supplemented with 20 µM Desferal, or minimal essential medium (MEM) supplemented with 20 mM sodium pyruvate. 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.

**Construction of C. jejuni fecB mutants.** The C. jejuni NCTC 11168 chromosomal DNA was extracted by using the Wizard genomic DNA purification kit (Promega). An internal fragment of the fecB gene was PCR amplified by using the primers fec1 and fec2 (both containing a MluNI restriction site; Table 2). The resulting 1,617-bp PCR product was digested with MluNI and ligated to the primers feo1 and feo2 (both containing a MluNI restriction site; Table 2). An internal fragment of the slyD homolog, we reevaluated and further studied the function of FeoB in ferrous iron acquisition. Here, we present the role of FeoB in C. jejuni iron acquisition, gut colonization, and intracellular survival.

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**RNA extraction and operon mapping by reverse transcription-PCR (RT-PCR).** Total RNA was extracted from C. jejuni NCTC 11168 grown to mid-log-phase in iron-restricted MEMs using a hot phenol-chloroform protocol as previously described (20, 28, 30). After total RNA extraction, RNA was suspended in RNase-free water and subjected to two successive DNase I (Invitrogen) treatments to remove any contaminating chromosomal DNA. Finally, RNA was further purified using an RNeasy kit (QIAGEN, Valencia, CA). The absence of genomic DNA was ensured by PCR using the fec1 and fec2 primers. The RNA integrity was confirmed by agarose gel electrophoresis, followed by quantification with RiboGreen RNA quantification reagent (Molecular Probes). The purified total RNA was stored at −80°C until further use.

The cotranscription of Cj1395, fecO (Cj1397), and fecB (Cj1398) was assessed by using 100 ng of DNase-treated total RNA and a combination of six primers (Table 2 and Fig. 1). First-strand cDNA synthesis and subsequent PCR amplification were performed by using the QIAGEN One-Step RT-PCR kit according to the manufacturer's recommendations and as previously described (20, 28, 30). The RT-PCR products were separated on 0.9% agarose gel with the 1-kb standard DNA ladder (Bayou-Biolabs).

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR was performed by using the ABI Prism 7500 DNA analyzer (Applied Biosystems, Foster City, CA) and the QuantiTect SYBR green RT-PCR kit (QIAGEN) according to the manufacturer's protocol and as previously described (20, 28, 30, 31). Total RNA was extracted from C. jejuni wild-type and fecB mutant strains grown to mid-log phase in iron-restricted MEMs as described above for the operon mapping by RT-PCR. The relative expression level of the fecO gene in the wild-type and fecB mutant strains was normalized to either shrD (encoding the

**FIG. 1. Operon mapping by RT-PCR analysis.** The predicted RT-PCR products within the Cj1395-to-Cj1399c genomic region are shown at the bottom. The RT-PCR product labels match the numbering of the agarose gel lanes. Lanes 1 and 7 correspond to the 1-kb ladder. The template RNA was extracted from C. jejuni NCTC 11168 cells grown in MEMs.
peptidyl-prolyl cis-trans isomerase) or hIC (encoding the ketol-acid reducto-
iserase). The expression of both shD and hIC was previously shown to be invar-
tant to the iron content of the growth medium. The primers used are listed in
Table 2. Quantitative values were obtained by using the comparative threshold
cycle (ΔΔCT) method, as recommended by Applied Biosystems and as previ-
ously described (20, 28, 30, 31). The feoB transcript level from each RNA sample
was assayed three times, and the mean ΔCt value was used for further analysis.

Ferric iron uptake experiments. Wild-type and mutant strains of C. jejuni
were grown to mid-log-phase under microaerophilic conditions in MH, MH-
desferrirrioxamine (20 μM), or MEMN. The cells were centrifuged at 6,000
rpm for 15 min at 4°C, washed with 10 mM Tris buffer (pH 7.4), resuspended
in the uptake buffer (5 mM NaH2PO4, 5 mM KH2PO4, 1.18 g of NaHCO3, 0.089 g
of Na2SO4, 0.042 g of MgCl2, 6.9H2O, and 10 g of Casamino Acids/liter) to an
optical density at 600 nm (OD600) of 0.6 (equivalent to 105 CFU/ml), and kept on
ice. Then, 10 ml of the bacterial suspension was incubated at 37°C for 10 min, and
ferrous iron transport assays were started with the addition of 0.037 μM [55Fe]2+.
The [55Fe]2+ stock solution was prepared in the uptake buffer and contains 3.7 μM
[55FeCl3] (Perkin-Elmer, Boston, Mass.) and 60 mM sodium ascorbate to reduce
iron. Samples of 1 ml were drawn at appropriate times, and the cells were
immediately pelleted at 4°C by centrifugation at 13,000 rpm for 2 min. The
pelleted cells were washed twice with 2 ml of 0.1 M citrate and resuspended in
500 μl of cold water. Incorporated iron was determined by liquid scintillation
counting as previously described (29). The uptake assays were repeated at least
three times, and the data were statistically analyzed by using the Student's t test at
a 5% level of significance.

Determination of cellular iron accumulation. C. jejuni feed mutants and
wild-type strains were grown in 25 ml of MEM supplemented with 20 mM
sodium pyruvate (pH 7.2) (prepared as described for the iron uptake ex-
periments) at 37°C under microaerophilic conditions. Growth of the bacterial
cultures was monitored by measuring the OD600. The cellular accumu-
lation of [55Fe]2+ was determined by drawing a 1-ml aliquot of the culture after 0,
8, 12, and 24 h of bacterial growth. The cells were centrifuged at 13,000 rpm for
2 min, washed twice with 0.1 M citrate and resuspended in 500 μl of deionized
water. Intracellular iron was determined by liquid scintillation counting as described above. Housley et al. (44) determined the iron content of the periplastic
fractile colonization assays, each chick was inoculated with 0.5 ml of a bacterial suspension containing a 1:1 mixture of C. jejuni NCTC 11168 and
its feoB mutant at 107 CFU/ml. The bacterial titer and the one-to-one ratio
of the initial inoculum were confirmed by plating serial dilutions of the cultures
on MH agar plates. The bacterial titre was determined and expressed as CFU per gram of ceca. A nonparametric Mann-Whitney rank sum test was used for statistical analysis at a
5% level of significance.

Invasion assay into INT-407 and IPEC-1 cells. Human INT-407 embryonic
intestinal cells were obtained from the American Type Culture Collection and
routinely maintained in MEM, supplemented with 10% feto-vine serum (Invitro-
gen). Porcine IPEC-1 small intestinal epithelial cells were obtained from
H. M. Berschneider (North Carolina State University) and were routinely main-
2
ained on Dulbecco minimum essential medium (Invitrogen), supplemented with
5% fetal bovine serum (Invitrogen), insulin (5 μg/ml), transferrin (5 μg/ml),
serum (5 ng/ml) (Invitrogen), and epidermal growth factor (5 ng/ml; Invitro-
gen). Cells were grown in an incubator at 37°C under 5% CO2.

The binding and invasion assays were carried out as previously described (24).
Briefly, the binding assay was performed by coincubation of C. jejuni cells
grown to mid-log phase in biphase MH media with 24-hour-grown, semiconfluent INT-407
cells or 48-hour-grown IPEC-1 cells (∼106 cells per well) at a multiplicity of infec-
tion of 10:1 (10 bacteria per eukaryotic cell). After 3 h of incubation at 37°C in
the presence of 5% CO2, the cell growth medium was removed, and the mono-
layer was washed thrice with Hank's balanced salt solution (HBSS). Thereafter,
the infected cells were lysed with 0.1% Triton X-100 at room temperature for 30
min. Serial dilutions of the cell lysates were plated on MH agar plates to
enumerate the number of bacteria bound to and internalized in the eukaryotic
cells. For the invasion assay, the infected cells were incubated for an additional
hour in fresh medium containing 250 μg of gentamicin per ml to kill the extra-
cellular bacteria. Thereafter, the cells were washed three times with HBSS and
lysed by using 0.1% Triton X-100 for 30 min at room temperature. The number of
intracellular bacteria was determined by serial dilutions and enumeration on
MH agar plates. The binding efficiency was obtained by subtracting the number
of intracellular bacteria from the total number of bacteria recovered from cells
not subjected to gentamicin treatment. The invasion efficiency was expressed as
the percentage of inoculum recovered after gentamicin treatment. Each binding
and invasion assay was repeated at least three times, and the data represent the
means ± the standard errors. The data was statistically analyzed by using the
Student's t test at a 5% level of significance.

Determination of intracellular survival. The intracellular survival (ICS) assay
was performed with semiconfluent INT-407 and IPEC-1 intestinal epithelial
cells. After bacterial invasion and gentamicin treatment as described above for
the binding and invasion assays, the eukaryotic cells were washed three times
with HBSS and cultured for an additional 72 h in fresh MEMs supplemented
with 10% fetal bovine serum. This medium was changed every 24 h. After
the desired incubation time points (24, 48, and 72 h), and the monolayer was washed
three times with HBSS and lysed by using 0.1% Triton X-100 at room temperature
for 30 min. The number of viable intracellular bacteria was determined by plating
serial dilutions of the cell lysate suspensions on MH agar plates. The experiment
was repeated three times, and the results shown are the mean ± the standard
error. A Student's t test was used for statistical analysis at a 5% level of signifi-
cance.

Chick colonization model. One-day-old specific-pathogen-free chicks
were obtained from Tyson Farms in Arkansas. On arrival, cloacal swabs of the
chicks were taken (100) to ensure that the birds were free from the target
bacterial (prepared as described for the iron uptake experiments) at 37°C under
microaerophilic conditions. Growth of the bacterial cultures was monitored by
measuring the OD600. The cellular accumu-
lation of [55Fe]2+ was determined by drawing a 1-ml aliquot of the culture after 0,
8, 12, and 24 h of bacterial growth. The cells were centrifuged at 13,000 rpm for
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on MH agar plates. The bacterial titre was determined and expressed as CFU per gram of ceca. A nonparametric Mann-Whitney rank sum test was used for statistical analysis at a
5% level of significance.

For the chick competitive index, the chicks were euthanized, the ceca were collected, and the
contents were homogenized and checked for C. jejuni viable counts by plating
serial dilutions on broth plates of Campylobacter agar base (Oxoid CM935) containing
the Campylobacter selective Karimali antimicrobial supplements (Oxoid SR167E).
Plates were incubated at 37°C for 72 h, and the bacterial
recovery titer was determined and expressed as CFU per gram of ceca. A
nonparametric Mann-Whitney rank sum test was used for statistical analysis at a
5% level of significance.

For the chick competitive index, the chicks were euthanized, the ceca were collected, and the
contents were homogenized, serially diluted in PBS buffer, and then plated onto Campy-
lobacter selective agar plates, as described above, with or without kanamycin (30 μg/ml).
The plates were then incubated at 37°C for 72 h in a microaerophilic chamber before the colonies were counted. The mutant titer was obtained from the
CFU recovered on broth 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 broth plates without kanamycin. Finally, the in vivo competitive index was calculated for each bird, which is the ratio of mutant
to wild-type bacteria recovered (output ratio) divided by the ratio of mutant
to wild-type bacteria inoculated (input ratio). The data were statistically analyzed
by using a Student's t test at a 5% level of significance.

Neonate piglet infectious model. Colostrum-deprived neonatal piglets were
obtained from the swine farm of Oklahoma State University. The piglets were
checked upon arrival with rectal swabs to verify that they were Campylobacter
free. Piglets were group housed (segregated in experimental and control groups) in
electric steel swing pens with elevated vinyl coated floors. An ambient room
temperature was maintained between 26 and 29°C with supplemental heat pro-
vided by one or more heat lamps to provide 32 to 35°C directly under the heat
lamp(s). Room light timers were set to provide a 12-h dark/12-h light diurnal
light cycle. Piglets were fed a milk replacer (Multi-Purpose milk replacer, Grade
A Ultra244; Sav-A-Caf Products) four times daily. This milk replacer was warmed to ca. 84 to 86°F prior to feeding. The initial feeding was within 60 min of birth. For the first day (24 h) piglets were either bottle-fed or fed using a 60-ml syringe.

Subsequently, piglets were fed by placing milk replacer in shallow pans. Piglets were fed ca. 2 to 4 oz. (60 to 120 ml) per feeding. For the colonization assays, the 1- to 2-day-old piglets from the experimental groups were orally inoculated with a 29-ml mid-log-phase suspension of one of the three C. jejuni wild-type strains in Similac milk containing between 1010 and 1011 organisms per ml. Piglets were observed daily for clinical signs of disease (diarrhea, blood, and mucus in the stool) over a period of up to 3 days. The presence of blood in the feces was tested by using the EZ detection kit (Biomerica). Piglets were sacrificed at 3 days postinoculation. Any animal showing severe signs of debility (loss of strength, high fever, difficulty breathing, and/or no appetite), particularly if death appeared imminent, was immediately euthanized. Postmortem pathological changes of the gastrointestinal tract were recorded (e.g., hyperemia, edema, hemorrhage, or distention with gas). Immediately after euthanasia, necropsy was performed, and the duodenum, jejunum, ileum, colon, and cecum were independently excised and processed for histopathological analysis and C. jejuni enumeration as previously described (30).

For the in vivo competition assay, each feoB mutant and its wild-type strain were mixed at a one-to-one ratio (~5 × 1010 CFU per strain). The titers of the mutant and wild-type strain was determined, and the competitive index was computed and statistically analyzed as described earlier for the chick competitive colonization assay.

RIL model. The in vivo competitive index of the feoB mutant and the wild-type NCTC 11168 in the rabbit ileal loop (RIL) model was determined as previously described (30). Briefly, two 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. The four ileal loops were confirmed by plating serial dilutions of this mixed culture on MH agar with 5% sheep blood. The loops of two additional rabbits were inoculated with sterile PBS buffer and served as uninfected controls. After inoculation, the exteriorized intestinal loops were repositioned in the abdominal cavity, and the abdominal wall and skin were finally closed as per standard procedures. At 48 h postinoculation, the rabbits were anesthetized again, the intestinal loops were excised intact, and the animals were euthanized. The contents of the loops (including the mucous layer) were homogenized in 10 ml of PBS buffer and serial diluted. The titers of the feoB mutants and the wild-type strains were determined as described above for the chick and piglet animal models. Finally, the in vivo competition index for each loop was calculated, and the data were statistically analyzed by using the Student t test at a 5% level of significance.

RESULTS

Sequence analysis, operon mapping, and mutation of C. jejuni feoB. The analysis of the C. jejuni NCTC 11168 genome reveals the presence of two genes, Cj1397 and feoB, whose products share significant identities at the amino acid level with the E. coli FeoA (16%) and FeoB (29.5%) proteins, respectively (21). The C. jejuni FeoB protein has 50.5% identity with the H. pylori FeoB and is predicted to be an integral cytoplasmic protein of 613 amino acids, containing 10 potential transmembrane a-helices and an ATP/GTP binding site at the N-terminal region. Cj1397 is predicted to encode a protein similar in size to the E. coli FeoA (77 amino acids) and contains the FeoA conserved protein domain as shown by RPS-BLAST analysis (17). The feoA and feoB genes appear to be in an operonic structure and are divergently transcribed from Cj1399c (Fig. 1), which encodes a probable Ni/Fe-hydrogenase small subunit. The open reading frame upstream of feoA, Cj1395, is a pseudogene which encodes an hypothetical protein of unknown function. In order to experimentally demonstrate the cotranscription of feoA (Cj1397) and feoB genes, we performed RT-PCRs with primers that anneal within and across pairs of the Cj1395, feoA, and feoB genes (Fig. 1). Each RT-PCR gave a product of the expected size, demonstrating the operonic structure of the feoA (Cj1397) and feoB genes.

To determine the function of FeoB in the C. jejuni ferrous iron acquisition, we constructed a feoB mutant by marker exchange mutagenesis (see Materials and Methods) into three strains of C. jejuni, the genome sequenced strain C. jejuni NCTC 11168, and the two clinical isolates C. jejuni 81-176 and C. jejuni ATCC 43431. Analysis of the recently released genome information of C. jejuni 81-176 at the NCBI database reveals the presence of the feoA-feoB operon with the same genetic organization as in C. jejuni NCTC 11168. Although the genome of C. jejuni ATCC 43431 has not been sequenced, we have recently characterized its gene content by DNA and shotgun microarray analysis. This study indicated the presence of both feoA and feoB genes (23, 24).

We have been unable to complement the feoB mutants using the shuttle vector pRY112. Consequently, to confirm that the feoB mutation was nonpolar, the level of expression of feoA was quantified by real-time RT-PCR in the three wild-type strains and feoB mutants. To note, the gene downstream of feoB, Cj1399, is divergently transcribed from feoB in both C. jejuni NCTC 11168 and 81-176 strains (Fig. 1), and thus its expression level will not be affected by the mutation. The genetic organization of the feoAB operon in C. jejuni ATCC 43431 is unknown. Consequently, we could not assert whether the expression of the gene downstream of feoB will be affected by the feoB mutation in C. jejuni ATCC 43431. The expression level of the feoA gene was found to be increased in the feoB mutants (six- to eightfold) compared to their wild-type strains. This result indicates that the insertion of the kanamycin cassette into the feoB gene does not destabilize the feoAB transcript. Consequently, the mutation is nonpolar. In addition, the increase of the feoA expression level in the feoB mutants suggests that the feoAB operon expression is tightly regulated.

Uptake of ferrous iron. In order to assess the role of FeoB in ferrous iron acquisition, we compared the capacity of the wild-type and feoB mutant of C. jejuni NCTC 11168 to uptake ferrous iron (Fig. 2). The 55Fe iron uptake assay was performed in the presence of sodium ascorbate in order to maintain the ferrous reduction state and using C. jejuni cells grown to mid-log phase in iron-restricted medium (MH broth supplemented with 20 μM Desferal as previously described [20]). As shown in Fig. 2, the C. jejuni NCTC 11168 cells accumulate up to 7.5 pmol of ferrous iron per OD600 upon 15 min incubation with 55Fe2+. In contrast, the feoB mutant cells are significantly affected and incorporate only 0.8 pmol of ferrous iron per OD600 over the assay period (P < 0.05). This 10-fold decrease in ferrous iron accumulation indicates that FeoB has a critical role in iron acquisition.

Because the FeoB protein is predicted to be an integral protein involved in the transport of ferrous iron across the cytoplasmic membrane, the iron accumulated by the feoB mutant could be solely localized in the periplasmic space. To investigate the cellular localization of ferrous iron upon incubation with 55Fe2+, the bacterial cells were osmotically shocked (using the PeriPreps periplasmatic kit from Epicenter) and the periplasmic and cytosolic fractions were collected. Exposure of gram-negative bacteria to osmotic shock has been previously shown to result in the formation of spheroplasts and...
the substantial release of proteins and solutes from the periplasmic space (5). The formation of C. jejuni spheroplasts was confirmed by electron microscopy (data not shown). Although the electron microscopy could not assert the complete absence of cytosolic release, it did confirm the formation of significant breaks into the outer membrane, indicating that periplasmic solutes would be discharged upon such treatment.

Figure 2 shows that up to 90% of the transported iron accumulates within the cytosol of both the wild-type and the feoB mutant strains. This result indicates that the iron accumulated by the feoB mutant under these conditions is not blocked into the periplasmic space but is still transported into the cytoplasm via a non-FeoB-dependent mechanism.

**Growth characteristics and accumulation of iron.** To determine whether ferrous iron acquisition plays an important role in the growth of C. jejuni, we compared the ability of C. jejuni NCTC 11168 wild-type and feoB mutant strains to grow in MEMα with or without added ferrous iron at a 0.036 μM final concentration. While the MEMα contains trace amount of iron, it has been previously used by us and others as an iron-limited growth medium (20, 34). The growth yield of the feoB mutant is slightly lower than the wild-type strain in MEMα with or without added ferrous iron (A, Fig. 3A, closed and open symbols, respectively), suggesting that the FeoB protein promotes bacterial growth. Interestingly, the addition of 0.036 μM ferrous iron to the MEMα stimulates the growth of the wild-type strain only. This observation is in agreement with the role of FeoB as a major route for ferrous iron acquisition.

To monitor cellular iron accumulation, the wild-type and feoB mutant strains were grown in MEMα supplemented with 0.036 μM 55Fe2⁺, and the levels of intracellular iron were quantitatively determined at various time points during growth.
similar survival characteristics during the first 48 h. At 72 h efficiencies were also indistinguishable between both strains of 10 to 1 for 3 h, and the survival kinetics were analyzed over a 72-h period. Campylobacter cells were grown in MH medium before infection. The first datum point at time zero hour represents the bacterial inoculum (10^9 bacteria). The experiment was repeated thrice and the data represent the mean ± the standard error.

by liquid scintillation counting. As shown in Fig. 3B, the feoB mutant accumulates, at the stationary phase, the labeled ferrous iron to an extent of 1.5-fold lower than the wild-type strain. Interestingly, the analysis of intracellular iron localization (as described above) indicates that the iron accumulated in the feoB mutant is essentially localized in the periplasmic space (80% of the total accumulated iron; Fig. 3C). In contrast, the wild-type strain accumulates 87% of its iron content into its cytosol (Fig. 3C). To note, iron accumulation in the periplasmic space of the feoB mutant was not observed during the iron uptake experiment (Fig. 2) likely as a result of the short 15-min incubation time with labeled ferrous iron under this experimental setting. In fact, the total amount of labeled iron accumulated in the feoB mutant is 30-fold lower during the uptake experiment compared to the accumulation assay. Altogether, these data indicate that C. jejuni FeoB is the major route for the transport of ferrous iron across the cytoplasmic membrane. This conclusion is in agreement with the annotation of FeoB as an integral membrane protein.

Intracellular survival of the C. jejuni feoB mutant in INT-407 and IPEC-1 cells. To understand the contribution of ferrous iron acquisition for survival in eukaryotic cells, we tested the ability of the C. jejuni feoB mutant to survive intracellularly up to 72 h. Given the low efficiency of C. jejuni NCTC 11168 to invade eukaryotic cells (24), these assays were performed with the wild-type and feoB mutant strains of C. jejuni 81-176, previously shown to have a high invasion efficiency (23).

Human INT-407 embryonic intestinal cells and porcine IPEC-1 small intestinal epithelial cells were infected with the wild-type and feoB mutant strains at a multiplicity of infection of 10 to 1 for 3 h, and the survival kinetics were analyzed over 72 h (Fig. 4). The integrity of the cell monolayers was maintained by changing the growth medium every 24 h and was checked under an inverted microscope. The wild-type and feoB mutant strains exhibit similar invasion efficiencies with ca. 0.6% of the inocula invading the intestinal cells. The binding efficiencies were also indistinguishable between both strains (data not shown). As illustrated in Fig. 4, both strains exhibit similar survival characteristics during the first 48 h. At 72 h postinfection, the feoB mutant is significantly affected in its ability to persist within INT-407 and IPEC-1 cells (P < 0.05). It is approximately five- and eightfold more sensitive than the wild-type strain in its ability to survive within IPEC-1 and INT-407 cells, respectively (Fig. 4A and B). These data indicate that FeoB plays an essential role in C. jejuni intracellular survival. Consequently, ferrous iron might constitute an important iron source in the eukaryotic intracellular environment.

Colonization of the chick cecum. To examine the role of FeoB in C. jejuni colonization, the wild-type and feoB mutant strains of C. jejuni NCTC 11168, 81-176, and ATCC 43431 were tested in the chick animal model of colonization as previously described (20). Each mutant and wild-type strain was orally administered to five birds at a dose between 10^4 and 10^5 viable bacteria, and the level of bacterial colonization in the chick cecum was evaluated 4 days postinoculation by plate counting. To note, the isolate of C. jejuni 81-176 used in the present study is a relatively poor colonizer of the chick cecum compared to the two other strains, since it colonizes the cecum at a level of approximately 10^5 CFU per g compared to ~5 × 10^7 for the two other strains of C. jejuni (Fig. 5). As shown in Fig. 5, the three feoB mutants are significantly affected in their ability to colonize the chick cecum compared to their respective wild-type strains (P < 0.05, using a nonparametric Mann-Whitney rank sum test). These data indicate that FeoB significantly promotes C. jejuni colonization of the chick cecum.

Interestingly, the feoB mutants of C. jejuni 81-176 appear to be less affected than the feoB mutants of C. jejuni NCTC 11168 and ATCC 43431 in its ability to colonize the chick cecum (Fig. 5). The feoB mutants of C. jejuni ATCC 43431, NCTC 11168, and 81-176 showed approximately (5 × 10^4)-fold, (2 × 10^3)-fold, and 31-fold decreases in their colonization ability compared to their corresponding wild-type strains, respectively. This observation suggests that C. jejuni strains differ in their requirement for iron and/or their capacity to acquire iron in vivo.

Colonization of the rabbit ileal loop. We have recently shown by genome-wide expression profiling that the C. jejuni NCTC 11168 feoB gene is expressed in vivo during host colo-
C. jejuni wild-type strains NCTC 11168, 81-176, and ATCC 43431 and their derivative feoB mutants. Sets of five chicks were fed $10^3$ to $10^8$ cells of one of the wild-type strains C. jejuni or their derivative feoB mutants. At 5 days postinoculation the ceca were recovered, homogenized, serial diluted, and plated on selective medium for C. jejuni enumeration as previously described. Each point represents the colonization level (in CFU per gram of cecum) of each strain from a single chick. The dash line indicates the limit of detection, and the bars indicate the median bacterial colonization level.

FIG. 5. Colonization of the chick cecum by C. jejuni wild-type strains NCTC 11168, 81-176, and ATCC 43431 and their derivative feoB mutants. The competitive index of the feoB mutant is not affected.

In order to further study the mechanism of C. jejuni NCTC 11168 iron acquisition in the intestinal tract of rabbit, we tested the ability of the feoB mutant to colonize the rabbit ileal loop relative to the wild-type strain in a competition assay. The feoB mutant and the wild-type strains were pooled together at a 1-to-1 ratio and inoculated into four ileal loops constructed in two different rabbits. After 48 h postinoculation, the ileal loops were recovered and processed for bacterial enumeration by plating the loop contents on selective media. Then, the in vivo competitive index was calculated by dividing the number of feoB mutant strains recovered by the number of wild-type strains recovered. Similarly, we determined the in vitro competitive index at the stationary phase by inoculating MH broth with an equal amount of the wild-type and the feoB mutant strains (the in vitro experiment was performed in triplicate). As shown in Fig. 6, the feoB mutant exhibits an in vivo and in vitro competitive index of 0.46 ± 0.11 and 1.00 ± 0.05, respectively. This 2.2-fold difference was found to be statistically significant using a Student t test ($P < 0.01$). These data indicate a slight growth and/or survival defect of the feoB mutant in the rabbit ileal loop.

In order to directly compare the colonization ability of the feoB mutant in the rabbit ileal loop and chick models, we determined the competitive index of the feoB mutant in the chick model. The competitive index of the feoB mutant in this animal model is $(1.4 \times 10^{-5}) \pm (7.3 \times 10^{-5})$ (Fig. 6), which is significantly lower than in the rabbit ileal loop model ($P < 0.0001$). This difference in the competitive indices likely reflects the presence of more severe bottlenecks in the chick competitive colonization assay versus the rabbit ileal loop model. In the rabbit ileal loop model, once the feoB mutant and the wild-type strains are introduced in the intestinal loop, they will have to compete essentially only for growth nutrients and survival under these conditions. In contrast, in the chick competitive colonization assay, the mutant and wild-type strains will have to survive under more stressful conditions. The presence of bottlenecks in the chick colonization process has been described by others (10) and likely accounts for the difference in the competitive indexes observed in our two colonization assays.

Colonization of the piglet intestine. To investigate whether the C. jejuni feoB mutant was defective in colonization in an animal model that mimics human infection, competition assays were performed in the newborn piglet model for campylobacteriosis (2).

Given that the level of C. jejuni colonization in this animal model has never been reported, we tested the ability of the three strains of C. jejuni used in the present study to colonize the gastrointestinal tract of newborn piglets and to induce the development of diarrhea and clinical signs of disease. Groups of three newborn colostrum-deprived piglets were orally challenged with a bacterial suspension of either C. jejuni NCTC 11168, 81-176, or ATCC 43431 at a dose of $5 \times 10^{10}$ viable bacteria. An additional group of three noninfected piglets was kept as control. In contrast to the method described by Babakhani et al. (2), the piglets were fed a multipurpose milk replacer (Sav-A-Caf) instead of Similac (Ross Laboratories,
Columbus, Ohio), because in the course of pilot studies we have noticed that Similac feeding induces the development of soft feces and protein deficiency. Piglets were observed for clinical signs of disease over a period of 3 days. The piglets infected with the strains of *C. jejuni* developed diarrhea 12 to 24 h postinoculation (Table 3). No diarrhea was observed in the piglets from the control group. Interestingly, the presence of blood was solely identified in the loose feces of animals infected with *C. jejuni* ATCC 43431 and 81-176 and not those infected with *C. jejuni* NCTC 11168 (Table 3). After 3 days of infection, most of the infected piglets showed signs of debility precluding the extension of this animal experiment beyond 3 to 4 days of infection. Consequently, the piglets were euthanized, and the level of bacterial colonization was determined for each intestinal segment (duodenum, jejunum, ileum, cecum, and colon). The data represent the mean CFU for each strain per gram of tissue.

### TABLE 3. Piglet infectious model

<table>
<thead>
<tr>
<th>Site</th>
<th>Control</th>
<th>Mean CFU/g of tissue (SD) for <em>C. jejuni</em> strain*:&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCTC 11168</td>
<td>ATCC 43431</td>
</tr>
<tr>
<td></td>
<td></td>
<td>81-176</td>
</tr>
<tr>
<td>Duodenum</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 × 10&lt;sup&gt;5&lt;/sup&gt; (2.5 × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>ND</td>
<td>6.2 × 10&lt;sup&gt;4&lt;/sup&gt; (4.5 × 10&lt;sup&gt;4&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Ileum</td>
<td>ND</td>
<td>9.3 × 10&lt;sup&gt;4&lt;/sup&gt; (4.6 × 10&lt;sup&gt;4&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Cecum</td>
<td>ND</td>
<td>1.5 × 10&lt;sup&gt;3&lt;/sup&gt; (9.1 × 10&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Colon</td>
<td>ND</td>
<td>2.8 × 10&lt;sup&gt;6&lt;/sup&gt; (1.3 × 10&lt;sup&gt;6&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Blood in feces&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Absent</td>
<td>Present</td>
</tr>
</tbody>
</table>

* Three colostrum-deprived piglets were infected orally with approximately 5 × 10<sup>8</sup> viable bacteria of each *C. jejuni* strain. At 3 days postinfection, the piglets were euthanized, and the level of bacterial colonization was determined for each intestinal segment (duodenum, jejunum, ileum, cecum, and colon). The data represent the mean CFU for each strain per gram of tissue.

<sup>a</sup> ND, *C. jejuni* was not detected in the control piglets.

<sup>c</sup> The presence of blood in the feces was tested using the EZ detection kit.

### DISCUSSION

Bacterial colonization of the host gastrointestinal tract depends in part on the ability of the bacteria to acquire essential growth nutrients. Iron is known to be a vital element for almost all living microorganisms (1). However, little is known about the bioavailability and sources of iron for enteric microbes in the gastrointestinal niche. Most of the iron that enters the intestine is in the oxidized ferric form. While the iron content of the intestinal environment is unknown. The role of ferrous iron acquisition in gut colonization is poorly studied. However, genome sequencing projects reveal that most enteric gram-negative bacteria are equipped with a Fe<sup>2+</sup> active transport system named Feo (1, 11). In addition, *feoB* mutants of *S. enterica* serovar Typhimurium, *H. pylori*, and *E. coli* have been shown to exhibit significant intestinal colonization defects (32, 33, 35). Altogether, these data indicate that ferrous iron might constitute an important iron source of the intestinal environment. Given that *C. jejuni* primarily resides within the host intestine, we significantly affected in their abilities to colonize the piglet intestine (*P* < 0.0001). The *feoB* mutant of *C. jejuni* ATCC 43431 is the most affected mutant, followed by the *feoB* mutant of *C. jejuni* 81-176 and then the *feoB* mutant of *C. jejuni* NCTC 11168. This difference in growth defect between the three *feoB* mutants indicates that the three strains of *C. jejuni* should significantly differ in their growth requirement for iron and/or their capacities, other than FeoB-mediated, to acquire iron. Interestingly, the colonization defect of the *feoB* mutant of *C. jejuni* ATCC 43431 varies within the gastrointestinal tract. The number of *C. jejuni* ATCC 43431 *feoB* mutant was below the detection limit in the duodenum, jejunum, ileum, and colon sections (<100 CFU/g). However, the *feoB* mutant of *C. jejuni* ATCC 43431 was recovered from the cecum, albeit at a much lower level than the wild-type strain (with a competitive index of 0.035 ± 0.030). These data indicate the presence of different iron sources and/or differential bacterial iron requirement within the gastrointestinal tract. Altogether, these in vivo competitive assays highlight a major role for FeoB in piglet gut colonization.
FeoB-MEDIATED FERROUS IRON ACQUISITION IN C. JEJUNI

FIG. 7. Competitive index analysis of the wild-type strains C. jejuni NCTC 11168 (A), ATCC 43431 (B), and 81-176 (C) and their respective feoB mutant in the gastrointestinal tract of piglets. Each wild-type strain and its respective feoB mutant were mixed at a ratio of 1 to 1 and inoculated into three piglets. At 72 h postinoculation, the piglets were euthanized, and the intestinal segments (duodenum, jejunum, ileum, cecum, and colon) were collected. The competitive index 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 represent the means ± the standard errors. An asterisk indicates that the number of feoB mutant per gram of intestine was below the detection limit of our assay (100 CFU/g), and consequently a competitive index was not calculated.

The analysis of the C. jejuni NCTC 11168 genome reveals the presence of a single potential FeoAB Fe\(^{2+}\) transporter system (21). In this report we provide many evidences that C. jejuni FeoB is part of a functional ferrous iron transporter system. First, C. jejuni NCTC 11168 FeoB is highly homologous to the E. coli and H. pylori FeoB proteins. Second, a feoB mutant of C. jejuni is significantly affected in its ability to transport ferrous iron (Fig. 2; \(P < 0.05\)). Third, the C. jejuni feoB mutant accumulates a lower amount of iron during growth compared to the wild-type strain (Fig. 3). Finally, the intracellular iron accumulated by the feoB mutant is localized in the periplasmic space compared to the cytosol for the wild-type strain (Fig. 3C), a result that is in agreement with the function of FeoB as a cytoplasmic membrane transporter.

Notably, our functional characterization of FeoB is in disagreement with the recent report from Raphael and Joens (25). In that report, the absence of Fe\(^{2+}\) uptake defect in the C. jejuni feoB mutant from Raphael and Joens could be the result of their experimental approach (25). Alternatively, the absence of observable ferrous iron uptake defect in the feoB mutant from Raphael and Joens is unknown, the ferrous iron uptake system of other bacteria has been shown to be repressed by iron. Consequently, the FeoB-mediated ferrous iron transport is unlikely to be functional in iron-loaded cells, as would be C. jejuni cells grown on MH plates. In contrast, we grew C. jejuni in iron-limited MEM media. Although the regulation of the feoB expression in C. jejuni is unknown, the ferrous iron uptake system of other bacteria has been shown to be repressed by iron. Consequently, the FeoB-mediated ferrous iron transport is unlikely to be functional in iron-loaded cells, as would be C. jejuni cells grown on MH plates. In order to test this possibility and address the results from Raphael and Joens (25), we compared the capacity of the wild type and feoB mutant of C. jejuni NCTC 11168 to transport ferrous iron by using cells grown on MH plates (data not shown). The feoB mutant of C. jejuni NCTC 11168 exhibited no defect in ferrous iron uptake under such growth conditions (data not shown), which is in agreement with the phenotype of the feoB mutants of C. jejuni M129 and F38011 (25). In summary, our data clearly demonstrate the role of FeoB in ferrous iron uptake in C. jejuni under iron-limited conditions and suggest that the FeoB transporter system might be iron regulated. The regulation of the feoB gene is currently under investigation.

The importance of ferrous iron acquisition for bacterial intracellular survival and growth is evinced by several examples. The feoB mutant of Legionella pneumophila exhibits impaired replication in amoeba and human U937 cell macrophages, highlighting an important role for the FeoB system in intracellular infection (26). While mutants of Shigella flexneri defective in a single iron transporter system—feoB, sitA (which encodes a component of an iron transporter system), or iucC (which
encodes a component of the aerobactin transporter system)—show no defect in intracellular growth, double mutations in the feoB gene and any one of the two other transporter systems (sitA or iucC) significantly decrease the capacity of S. flexneri to form plaque on Henle cell monolayers (27). These findings indicate that both ferrous and ferric iron acquisitions contribute to the intracellular replication of S. flexneri (27). Likewise, only the double mutant in feoB and sitA of S. enterica serovar Typhimurium was impaired in its ability to replicate intracellularly (4). Based on these observations, the ferrous iron appears to be an important intracellular iron source for pathogens. Similarly, our data indicate that FeoB-mediated Fe$^{2+}$ uptake is required for C. jejuni survival within human INT-407 embryonic intestinal cells and porcine IPEC-1 small intestinal epithelial cells. Although the intracellular persistence of the feoB mutant is similar to the wild-type strain during the first 48 h, it is inhibited for survival by approximately five- or eightfold at 72 h, depending on the cell lines (Fig. 4). This observation indicates that the amount of iron stored by the feoB mutant might be sufficient to persist within epithelial cells over the course of a 48-h incubation time and that the acquisition of iron via the FeoB system is only required for persistence past this initial stage. These findings add the feoB gene to the list of three other genes known to encode proteins contributing to intraepithelial or intramacrophage cell survival. These genes are spoT encoding a bifunctional (p)ppGpp synthetase/hydrolase (9), katA encoding a catalase (7), and sodB encoding a superoxide dismutase (22). Cellular invasion and intracellular survival have been associated with C. jejuni virulence (13). Indeed, mutants affected in their invasion ability were also shown to exhibit reduced virulence in the ferret animal disease model (3). Consequently, the C. jejuni feoB mutant, in addition to its colonization defect, might have attenuated virulence potential. We have recently shown the expression of the C. jejuni feoB gene in rabbit ileal loops, suggesting an important role for FeoB in Campylobacter gut colonization and/or survival (30). To address this hypothesis, we compared here the ability of the feoB mutant and C. jejuni NCTC 11168 wild-type strain to colonize the rabbit ileum in a competitive assay as previously described (30). The feoB mutant exhibits a competitive index of 0.46 in vivo and 1.00 in vitro. These data suggest a modest, but statistically significant, in vivo growth defect for the feoB mutant. The relatively short infection time (48 h) and the physical ligation of the rabbit intestinal tract in this animal model likely underestimate the importance of FeoB-mediated ferrous iron acquisition for gut colonization, resulting in the observed modest in vivo colonization defect of the feoB mutant. Subsequently, to assert the importance of FeoB in vivo and to gain a better understanding of the role of FeoB in the colonization of the host gastrointestinal tract, we compared the capacities of our three feoB mutants and wild-type strains of C. jejuni to colonize the chick cecum and the piglet gastrointestinal tract. In the chick, where C. jejuni is a commensal organism, it does not induce any disease, and it colonizes the cecum at a high level after oral inoculation (20). Consequently, the chick animal model is the model of choice to study C. jejuni colonization abilities. However, given the numerous physiological differences between the bird and human gastrointestinal tracts, the information gathered from the colonization of the chick cecum might not be true for the colonization of the mammal intestine during infection. Thus, we also tested the colonization potential of our C. jejuni strains in the newborn piglet model of human campylobacteriosis (2). Since the porcine gastrointestinal tract and digestive physiology are very similar to those of humans, the newborn piglet constitutes a good model of human gastroenteritis. In addition, this animal model was previously shown to reproduce clinical outcomes associated with human Campylobacter infection (2). In summary, the use of both animals, the chick and the piglet models, should provide valuable information on the role of FeoB in the colonization of the gut during commensal and infectious relationships. As shown in Fig. 5, the feoB mutants of all three strains of C. jejuni are significantly affected in their ability to colonize the chick cecum, indicating an important role for ferrous iron acquisition in vivo. The fact that the feoB mutants are not entirely inhibited for colonization suggests that they can partially overcome the absence of ferrous iron uptake by acquiring iron from a different source. Analysis of the genome sequence of C. jejuni NCTC 11168 reveals that this strain harbors, in addition to the FeoAB ferrous iron transporter, at least two ferric-siderophore transporter systems (CfrA-CeuBCDE for the transport of ferric-enterobactin and Cj0178-Cj0173c/Cj0174c/Cj0175c for the transport of an unknown ferric-siderophore), one hemin transporter system (ChuABCD), and one additional putative iron acquisition system (Cj1658-p19). We have previously shown that the cfrA, ceuE, and Cj0178 mutants of C. jejuni NCTC 11168 were also significantly affected in their ability to colonize the chick cecum, highlighting the importance for siderophore-mediated iron acquisition in vivo (20). Altogether, these data indicate the requirement of both ferric-siderophore and ferrous iron transport systems for the colonization of the chick cecum by C. jejuni and suggest that both ferrous and ferric iron sources are available in the intestine. Interestingly, the extent of the colonization defect of the three feoB mutants varies significantly between them. The feoB mutants of C. jejuni NCTC 11168, ATCC 43431, and 81-176 are inhibited for colonization by approximately 20,000-, 50,000-, and 31-fold, respectively. The variation in the colonization defect between the three feoB mutants suggests that these strains differ in their iron requirement for in vivo growth and/or in their ability to acquire iron from other sources to overcome the absence of ferrous iron uptake. In agreement with this hypothesis, we and others have previously shown that these three strains of C. jejuni harbor a different set of iron uptake systems (8, 23, 24). In fact, the C. jejuni ATCC 43431 genome lacks the ferric-enterobactin transporter and C. jejuni 81-176 lacks two ferric-siderophore transporter systems (Cj0178 and CfrA). Consequently, whereas ferrous iron appears to be a common iron source for all three strains of C. jejuni, the primary source of ferric-siderophore is different between them. Finally, in order to address the function of FeoB-mediated ferrous iron acquisition for gut colonization in an infectious model of campylobacteraiosis, we first established the use of the neonate piglet model to study Campylobacter infections with our three wild-type strains, C. jejuni NCTC 11168, ATCC 43431, and 81-176. As shown in Table 3, the three wild-type strains of C. jejuni colonize the piglets at a level ranging from $10^3$ to $10^7$ CFU per g of intestinal section on day 3 postinocu-
lotion (ca. 10^{10} cells were inoculated orally into each piglet for each bacterial strain). All infected piglets developed diarrhea 12 to 24 h postinoculation. Histological examinations of the intestinal tissues did not reveal any significant pathological lesions. The lack of lesions might simply be a reflection of the fact that the infection period of 3 days was not sufficient to trigger severe inflammation, as previously observed by Babakhani et al. (2). Nevertheless, the induction of diarrhea in pigs infected with each of the three C. jejuni strains and the presence of blood in the feces of pigs infected with C. jejuni 81-176 and ATCC 43431 indicate that this animal model reproduces the clinical symptoms observed during human infections. The absence of blood in the feces of piglets infected with C. jejuni NCTC 11168 suggests that this strain might be less virulent than the other two strains of C. jejuni. Given that epithelial cell invasion has been shown to be an important aspect of Campylobacter pathogenesis (3), the lower virulence of C. jejuni NCTC 11168 would be in agreement with the lower ability of this strain to invade epithelial cells (24). The presence of blood in the feces of the piglets infected with C. jejuni 81-176 and ATCC 43431 suggests the existence of histopathological lesions. Strikingly, however, such lesions were not observed. Several causative factors could explain the lack of lesions. First, the infection period was too short to induce significant lesions. Second, the histopathological lesions occurred at intestinal sections that were not examined and/or the number of intestinal sections examined was insufficient. Third, the occurrence of blood in the stool is the consequence of intestinal hemorrhage by diapedesis upon C. jejuni infection.

In the piglet competition assay, each wild-type strain of C. jejuni outcompetes its respective feoB mutant, indicating that ferrous iron is an important iron source in the pig intestine and that FeoB-mediated iron acquisition is required for C. jejuni gut colonization during infectious relationship with its host. The feoB mutants of the three strains of C. jejuni appear to exhibit different colonization defects (Fig. 7). This outcome likely results from the presence of a different set of iron acquisition systems in these three strains, which is in agreement with the data obtained from the chic colonization assay. Strikingly, the feoB mutant of C. jejuni ATCC 43431 is more affected in its colonization potential in the duodenum, jejunum, ileum, and colon than in the cecum. In fact, the feoB mutant was only recovered in the cecum section of the intestine. In the four other intestinal sections, the number of feoB mutant per gram of intestine was below the detection limit of our assay (100 CFU/g). This observation suggests the presence of different iron sources along the gastrointestinal tract and that the feoB mutant of C. jejuni ATCC 43431 is unable to efficiently acquire these iron sources in the duodenum, jejunum, ileum, and colon sections of the intestine. In contrast, this feoB mutant appears to modestly surmount the lack of ferrous iron uptake in the cecum. Given that the colonization of the cecum is higher than in the other intestinal sections, it is tempting to propose that C. jejuni ATCC 43431 acquires iron in the cecum from a ferric siderophore source produced by the indigenous microflora.

In summary, we showed that the feoB gene from C. jejuni encodes a functional component of the ferrous iron acquisition system. Our data indicate that FeoB provides a selective ad-}

**REFERENCES**


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