The Ferrous Iron Transporter FeoB1 is Essential for *Clostridioides difficile* Toxin Production and Pathogenesis in Mice

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ABSTRACT

Ferrous iron is the dominant form of iron in the oxygen-limited large intestine, the site for *Clostridioides difficile* infection (CDI). We investigated the extent to which *C. difficile* requires the ferrous iron transporter 1 (FeoB1), a main permease for iron acquisition, using *in vitro* and *in vivo* approaches. Construction of *feoB1* deletion mutant in *C. difficile* R20291 (i.e., R20291 Δ *feoB1*) decreased intracellular iron content by ~25%. This was accompanied by reduced synthesis of TcdA and TcdB toxins and downregulation of *tcdA* and *tcdB* genes, which was reflected by a ~1000-fold reduction in cytopathology against Vero cells. Complementation with WT *feoB1* restored toxin production. R20291 Δ *feoB1* was avirulent in mice with underlying colitis. In this model, mice are prone to develop severe CDI, but mice infected with the mutant lacked diarrheal symptoms, had lesser inflammatory responses, bacterial bioburdens and cecal toxin titers. Transcriptional and biochemical analyses revealed Δ *feoB1* altered metabolic pathways that negatively impact toxin production, including downregulation of the oxidative branch of the Krebs cycle and an associated cellular accumulation of pyruvate. Hence FeoB1 influences multiple bioenergetic and redox pathways, which in turn inhibits toxin biosynthesis. Targeting FeoB1 could be a potential therapeutic strategy to impede the colonization and pathogenesis of *C. difficile*, even in the setting of colitis where CDI is more severe and intestinal bleeding may occur.

AUTHOR SUMMARY

Clostridioides difficile is a major cause of antibiotic associated diarrhea that result in significant morbidity and mortality in hospitals. The ability of *C. difficile* to cause disease and damage the gut is directly related to the production of toxin A and toxin B. Without these toxins, *C. difficile* is not virulent. There is need to identify drug targets and therapeutic strategies that prevent *C. difficile* from producing toxins A and B. For *C. difficile* to colonize the large bowel, it must acquire ferrous iron, which is thought to be the main form of iron within the oxygen-limited large bowel environment. We show that genetic deletion of the ferrous iron transporter FeoB1 stops *C. difficile* from producing significant amounts of toxins under *in vitro* conditions. Importantly, the mutant R20291 Δ feoB1 was avirulent when tested in a mouse model that favors the development of severe infections. Avirulence of R20291 Δ feoB1 was related to the strain having significant metabolic changes that negatively impact toxin production. For example, cells could not properly metabolize pyruvate, which became accumulated in cells; pyruvate is known to reduce the production of toxins A and B. Our study provides new insights into how FeoB1 affects *C. difficile* colonization and disease development, indicating FeoB1 could be a drug target to stop toxin production in this bacterium.

INTRODUCTION

Clostridioides difficile infection (CDI) is a leading cause of hospital-acquired diarrhea, with 30,600 and 20,500 in-hospital deaths in 2011 and 2017, respectively [1]. The main risk factor for CDI is prior antibiotic use, which causes dysbiosis [2]. Treatment with anti-*C. difficile* antibiotics, vancomycin, metronidazole or fidaxomicin, are associated with recurrence rates that may be $\geq 10\%$ or higher [3-5]. Understanding the pathophysiology of *C. difficile* provides a basis to discover alternative therapeutics to prevent or treat recurrent disease.

CDI symptoms range from mild-to-moderate diarrhea to severe pseudomembranous colitis, resulting from epithelial damage by *C. difficile* toxins TcdA and TcdB [6]. These toxins glycosylate GTPases (e.g., Rho, Rac, Cdc42) to block actin polymerization, causing cells to appear rounded in tissue cultures [6]. Genes (*tcdA* and *tcdB*) encoding TcdA and TcdB are chromosomally located in the pathogenicity locus (PaLoc) [6]. PaLoc encodes the alternative sigma factor TcdR, which activates transcription of *tcdA* and *tcdB*; the TcdR antagonist TcdC that represses *tcdA* and *tcdB*; and the holin-like protein TcdE that exports the toxins. TcdA and TcdB are produced in the late logarithmic and stationary phases of growth and are regulated by environmental and nutritional changes. For example, *tcdA* and *tcdB* are repressed by metabolizable sugars (e.g., glucose) and metabolism of amino acids like proline, cysteine, and branched-chain amino acids (BCAAs) [7-9]. Glucose activates catabolite repression whereby the global regulator CcpA binds upstream of promoters in PaLoc, particularly the *tcdR* promoter, to block transcription of *tcdA* and *tcdB* [7]. During logarithmic growth, high intracellular concentrations of GTP bind to the nutritional regulator CodY, which in turn binds to regions of the *tcdR* promoter [10]. Through an unclear mechanism, pyruvate represses transcription of *tcdA* and *tcdB* [9]. Conversely, butyrate enhances

the synthesis of toxins by an unknown mechanism that may be related to NAD⁺ regeneration via alternative fermentative pathways that produce butyrate [11]. These and other examples, reviewed by Martin-Verstraete et al. [12] and Bouillaut et al. [13], all point to there being a complex metabolic and regulatory network for *C. difficile* toxin production.

Within environments that are anaerobic and acidic, such as the gastrointestinal tract, iron mainly exists as ferrous iron [14]. Gut bacteria must therefore be able to obtain ferrous iron in the microaerophilic and anaerobic niches of the intestine. The high affinity ferrous iron transporter (Feo transporter) plays an essential role in iron acquisition by bacteria in the intestinal tract [14, 15]. The Feo transporter mainly consists of FeoA, a small cytoplasmic protein, and FeoB, the membrane-bound permease that transports ferrous iron into cells. While the specific role of FeoA is still unclear, it might interact with the cytoplasmic domain of FeoB to regulate ferrous iron transport. Iron is indispensable for *C. difficile* growth and pathogenesis [16, 17] as several essential redox-associated proteins require the iron containing cofactor ferredoxin (Fdx) or iron-sulfur clusters. *C. difficile* possesses three homologs of the FeoB permease, designated FeoB1, FeoB2 and FeoB3 [17]. Under iron-limiting conditions, *feoB1* is the most transcribed *feo* homolog [9, 16, 17]; hence, FeoB1 is thought to be the main permease for ferrous iron acquisition in *C. difficile*. However, it is unknown whether FeoB1-mediated iron acquisition is critical for production of TcdA and TcdB.

Clostridium perfringens encodes a single FeoB transporter, and its deletion reduced growth and impaired iron-dependent fermentation and expression of the virulence toxins phospholipase C and perfringolysin O [18]. However, the authors [18] reasoned that reduced toxin production in *C.*

perfringens resulted from the effects on growth. Conversely, in *C. difficile*, deletion of *feoBI* in non-toxigenic ATCC 700057 did not affect growth in Brain Heart Infusion (BHI) broth, but reduced iron content [19]. This is particularly interesting since iron deficiency in *C. difficile* disturbs several metabolic processes, including shifting cells from ferredoxin to flavodoxin-mediated metabolism; increasing intracellular concentrations of pyruvate and glucose [16, 19]; and enhancing proline reduction via Stickland metabolism [16]. We therefore investigated whether iron deficiency caused by loss of *feoBI* impacts *C. difficile* toxin production and pathogenesis. Herein, we show that deletion of *feoBI* markedly abolishes toxin production and pathogenesis of *C. difficile* in mice.

RESULTS AND DISCUSSION

Loss of *feoBI* impairs toxin production. R20291 was adopted as a model strain of epidemic ribotype 027 [20] to delete *feoBI* by allelic exchange to generate R20291 Δ *feoBI*; deletion was confirmed by PCR and Sanger sequencing (**Fig S1**). Growth of R20291 Δ *feoBI* in BHI broth was not substantially affected, when compared to WT R20291 (**Fig 1A**); BHI broth contained 0.5 ppm of iron. Measurement of intracellular iron content, by ICP-OES, revealed there was a ~25% reduction in iron content in R20291 Δ *feoBI* (0.087 ± 0.006 ppm) compared to the WT (0.116 ± 0.007 ppm) (**Fig 1B**; $p \leq 0.01$, by unpaired t-test). These results are comparable to *feoBI* deletion mutant of non-toxigenic ATCC 700057, which had a ~21% reduction in iron content without attenuated growth in BHI broth [19]. We next quantified toxins in the supernatants of 24 h cultures using an ELISA kit from tgcBIOMICS GmbH. Toxin concentrations in R20291 Δ *feoBI* for TcdA (5.37 ± 0.66 ng/ml) and TcdB (3.51 ± 0.48 ng/ml) were significantly lower ($p < 0.0001$ and $p < 0.001$, by unpaired t-test) than those produced by WT R20291 (19.65 ± 0.67 ng/ml; and 11.46 ± 0.69 ng/ml, respectively) (**Fig 1C**). Complementation of R20291 Δ *feoBI* with WT *feoBI*, ectopically

expressed from its own promoter, increased the synthesis of both toxins (10.42 ± 0.66 ng/ml; and 6.77 ± 0.18 ng/ml, for TcdA and TcdB, respectively (**Fig 1C**). Comparable results were attained in cytopathic assays that measure toxin-induced cell rounding of mammalian cells and detects picomolar amounts of toxins [21]. Against Vero cells, R20291 Δ *feoB1* had toxin titers of $\leq 10^1$, which was 1000-fold lower than the titers of WT cultures (10^4 toxin titer; **Fig. 1D**); the complemented strain (R20291 Δ *feoB1*_WT *feoB1*) had toxin titers of 10^3 to 10^4 . These findings were confirmed by automated morphometric analysis of cell rounding to avoid potential bias from manual visualization of cells (**Fig 1E**); the in-situ dilution of supernatants was $\sim 1:1000$ and rounding of Vero cells is more due to TcdB as it is more refractory to TcdA [22]. WT R20291, R20291 Δ *feoB1* and R20291 Δ *feoB1*_WT *feoB1* caused 88.70 ± 1.60 , 10.54 ± 0.52 and 52.02 ± 0.74 percentage cell rounding for three replicates, respectively. Taken together, Δ *feoB1* diminished intracellular iron content and restricted the biosynthesis of TcdA and TcdB, without affecting growth rates in R20291.

Deletion of *feoB1* attenuated virulence in mice with colitis CDI. *In vivo* pathogenesis of R20291 Δ *feoB1* was investigated in a reported murine model of colitis CDI [23] that we modified by using a lower concentration of dextran sodium sulfate (DSS; 1.5% w/v instead of 3% w/v) and administering antibiotics and DSS for 5 days, as oppose to 3 days (**Fig 2A**). These modifications reduced reported DSS-related morbidity and deaths [23]. Because this model is characterized by intestinal bleeding [23] it enabled assessment of R20291 Δ *feoB1* virulence in a setting that favors iron availability at the site of infection. We performed two independent experiments. Ceca from animals infected with R20291 were inflamed, whereas ceca from animals infected with R20291 Δ *feoB1* were like those from uninfected mice (**Fig 2B**). Infection with WT R20291 was lethal to C57BL/6 mice as half of sixteen mice survived. In contrast, all fifteen mice survived that

were infected with R20291 Δ *feoB1* (**Fig 2C**); $p=0.0003$ according to log rank test. Significant weight loss was only observed in animals infected with R20291 and is a common symptom of acute CDI in mice (**Fig 2D**); however, mice infected with R20291 Δ *feoB1* either gained weight or experienced marginal weight loss ($p=0.0009$ according to unpaired t-test). Animals that succumbed to infection with R20291 had endpoint spore bioburdens of $\sim 10^7$ CFU/ml that were significantly higher than bioburdens from animals that survived infection (i.e., ~ 2 logs [$\sim 99\%$] greater; $p<0.0001$ determined by unpaired t-test; **Fig 2E**). Animals infected with R20291 Δ *feoB1* had even lower bioburdens than the surviving animals infected with the WT R20291 (i.e., >1 log [$\sim 93\%$] higher; $p<0.0001$ determined by unpaired t-test; **Fig 2E**). As a quality control, cecal isolates from the respective animals were confirmed to be R20291 from PCR amplicons of *CDR20291_1801* on *Tn6192* transposon in R20291 and *tcdA*; R20291 Δ *feoB1* was further confirmed from the absence of *feoB1* (**Fig S2**). Furthermore, there was no difference in *in vitro* germination for R20291 and R20291 Δ *feoB1* (**Fig S3**).

Comparison of pathogenesis in mice, at fixed time points. As the bioburdens in surviving animals at day 10 strongly suggested R20291 Δ *feoB1* inadequately colonized the large intestine, we repeated our assessment at a shorter time point of 48 h. Several studies indicate that high fecal bioburdens occur within 48 h of CDI in C57/BL6 mice and that this timepoint is suitable for measuring inflammatory responses in CDI [24, 25]. Therefore, we compared the colonization and pathogenesis of R20291 Δ *feoB1* and R20291 over 48 h. After inducing dysbiosis with antibiotics and DSS, 6 mice/group (3 males and 3 females) were infected with each strain. None of the animals infected with either of the strains became moribund, but those infected with the WT had significant weight loss when compared to the pre-infected weights (**Fig 3A**; $p \leq 0.01$), whereas those infected

with the mutant gained weight ($p \leq 0.05$). Bioburdens of cecal contents revealed mice infected with R20291 Δ *feoB1* had almost 3-logs less bacteria, compared to mice infected with R20291 and this was supported by bioburdens in feces taken at 24 and 48 h post-infection (**Fig 3B, C**); unpaired t-test $p < 0.0001$). Regarding toxins, ceca from WT infected mice had toxin titers of 10^2 - 10^3 (per gram of cecal content), which was 1-2 logs (90-99%) greater than cecal toxin titers in mice infected with R20291 Δ *feoB1* (**Fig 3D**). Evaluation of inflammatory responses by qRT-PCR showed that expression of pro-inflammatory tumor necrosis factor-alpha (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and anti-inflammatory interleukin-10 (IL-10) were all significantly lower in mice infected with R20291 Δ *feoB1* (**Fig 3E**). While TNF- α and GM-CSF promote neutrophil recruitment in CDI, the upregulation of IL-10 may occur to repress TNF- α and GM-CSF [26]. Elevation of these three cytokines may be markers for CDI severity in the CDI colitis model [23]. Overall, these findings indicate that *C. difficile* requires FeoB1 to colonize its host and produce toxin-mediated disease symptoms.

***C. difficile* adjust its metabolism to compensate for lack of FeoB1.** Transcriptomics was applied to identify whether Δ *feoB1* altered the expression of metabolic pathways that affect toxin production. RNA sequencing (RNA-seq) was done on R20291 and R20291 Δ *feoB1* cultures taken at mid-logarithmic (OD=0.3), late-logarithmic (OD=0.6) and stationary phase (OD=0.9). Genes were significantly differentially expressed based on $p \leq 0.02$ and Log₂fold change (FC) ≥ 1.5 i.e., $FC \geq 2.8$ following analysis in CLC Genomics Workbench version 12; **Table S1** list the expression changes for all genes. **Table 1** highlights selected pathways/genes affected by Δ *feoB1*, including genes that were identified at a less strict criteria of Log₂fold change (FC) ≥ 0.58 i.e., $FC \geq 1.5$. Across the three growth phases, there were 435 common differentially expressed genes, of which

151 were upregulated and 284 were downregulated (**Fig 4A**). Gene Ontology analysis revealed *ΔfeoB1* mostly affected redox and flavin-dependent processes (**Fig 4B, C**). Other differentially regulated processes included carbohydrate metabolism, iron homeostasis, pathogenesis, flagellar proteins, cell wall biosynthesis genes (**Fig 4B, C**). Although our studies were done in BHI media, expression changes seen in our RNAseq are comparable to those reported for *C. difficile* CD630 *fur* mutant that was grown in low iron media [16]. As described below, we focused on analyzing gene signatures that are linked to iron metabolism and *C. difficile* pathogenesis.

Iron-responsive gene transcription. Iron starvation in *C. difficile* increases the transcription of flavodoxin (*fldx*), ferric uptake regulator (*fur*), riboflavin biosynthesis and alternate iron uptake transporters [16]. In R20291 Δ *feoB1* there was upregulation of the flavodoxin encoded by *CDR20291_1925*; riboflavin biosynthesis genes *ribB* and *ribD*; and downregulation of *CDR20291_2756*, encoding the iron storage protein bacterioferritin (**Fig 5**). Upregulation of flavodoxin and riboflavin biosynthesis genes reflect a metabolic shift to adopt flavin cofactors, to likely substitute for the iron-dependent ferredoxin. Furthermore, downregulation of bacterioferritin suggests that cells sought to increase cellular availability of iron, by decreasing its storage in bacterioferritin. Alternate iron acquisition transporters were also upregulated such as ferrichrome-specific ABC transporter genes (*fhuC* and *fhuD*) (**Fig 5**), the low-affinity zinc transporter ZupT and Feo2 genes (*feoA2* and *feoB2*) (**Table 1**). Increased expression of *zupT* also occurs in *C. difficile* CD630 under iron limiting conditions [27] but it is unknown whether ZupT transports iron as previously shown in *Escherichia coli* (20). Deletion of *zupT* in *C. difficile* was reported to impair colonization in mice, although its effect on toxin production was not described [28]. In iron-starved media, *C. difficile* CD630 WT and a *fur* mutant were reported to upregulate

polyamine biosynthesis genes, which led to the hypothesis that polyamines (e.g., spermidine) may participate in acquiring and storing iron [16, 27]. However, in our transcriptome results, polyamine biosynthesis genes were either downregulated or not significantly affected, but putative transporters (e.g., CDR20291_3365) for polyamines were upregulated (**Table 1**).

Relationship between FeoB1, pyruvate metabolism and toxin production. In R20291 Δ *feoB1* (**Fig 6A**) PaLoc genes were significantly downregulated, including *tcdA* and *tcdB*, the sigma factor *tcdR* and holin *tcdE* (**Table 1**). Thus, the low levels of TcdA and TcdB protein in supernatants from R20291 Δ *feoB1* directly correlate with repression of *tcdA* and *tcdB*. We recently reported that Δ *feoB1* and disruption of iron-sulfur cluster biosynthesis caused pyruvate accumulation in non-toxicogenic ATCC 700057 [19]. In R20291 Δ *feoB1* we also observed an accumulation of pyruvate throughout the three growth phases, when compared to R20291 (**Fig 6B**). This was especially pronounced in the stationary phase, where there was a 34.48% increase in pyruvate, compared to 7.84% and 11.94% in mid- and late-logarithmic cultures, respectively. Pyruvate feeds into multiple metabolic pathways, where it can be converted to formate by pyruvate-formate lyase (Pfl); lactate by lactate dehydrogenase (Ldh); acetyl-CoA by pyruvate-ferredoxin oxidoreductase (NifJ); or enter the Krebs cycle to be converted to oxaloacetate by pyruvate carboxylase (Pyc) or malate by malate dehydrogenase (Mdh) [29]. Our transcriptome data showed many of these genes were differentially regulated (**Table 1**). In cultures at OD ~0.6 *pflD* and *pflE* were increased by 3.09- and 3.39-fold respectively but were not significantly differentially expressed in stationary phase cultures. In contrast, it was reported [16] that *pfl* genes were downregulated in a *C. difficile* CD630 *fur* mutant, which also accumulated pyruvate in chemically defined low iron media; upregulation of *pfl* genes in our study may reflect differences in media used and types of mutants studied i.e.,

fur versus *feoB1*. Interestingly, *pyc* was more than two-fold downregulated in the transcriptome. *C. difficile* encodes a partial Krebs cycle in which pyruvate is fed into the oxidative branch by Pyc converting it to oxaloacetate, which is then converted to citrate, isocitrate and 2-oxoglutarate, with the last two steps catalyzed by aconitase (*CDR20291_0763*) and isocitrate/3-isopropylmalate dehydrogenase (*CDR20291_0764*). Oxaloacetate is thought to be metabolized to citrate by homocitrate synthase (NifV), encoded by *CDR20291_0762* [30]. These genes (*CDR20291_0762* to *CDR20291_0764*) were all downregulated in the transcriptome (-1.89 to 12.93-fold across the three growth phases [Table 1]). RT-qPCR of *pyc*, *CDR20291_0763* and *CDR20291_0764* confirmed their downregulation (Fig 6C). Thus, it is likely that downregulation of *pyc* and the oxidative branch of the Krebs cycle contributed to the build-up of pyruvate, which is known to negatively affect transcription of *tcdA* and *tcdB* [9].

Other pathways associated with regulating toxin production were also upregulated. Interestingly, cultures at OD ~0.6 showed statistically significant, but moderate, increases in transcription of *prd* operon genes *prdA*, *prdB*, *prdC* and *prdF* (1.46-1.61-fold) that encode proteins involved in the Stickland metabolism of proline (Table 1). Upregulation of Stickland enzymes is also consistent with there being a shift to energy generating and redox balancing processes, which may include enzymes that do not rely on ferredoxin [21]. Furthermore, activation of Stickland reduction of proline is associated with repression of toxin production [13].

CONCLUSION

Within the oxygen-limited colon, the Feo system is crucial for intestinal pathogens since Fe²⁺ is likely the dominant form of iron. This may explain why *C. difficile* *feoB1* is upregulated by >200-fold in mice [31]. Our study clearly shows that *feoB1* is necessary for *C. difficile* to effectively

colonize and cause disease in mice. Although FeoB1 may be the main permease for ferrous iron acquisition, its deletion did not affect *in vitro* growth of *C. difficile*. This is consistent with there being various pathways for *C. difficile* to generate energy in a complex culture media. Such pathways include fermentation of amino acids in the Stickland pathway in which the oxidation of an amino acid (e.g., cysteine) is coupled to the reduction of another (e.g., proline) to produce ATP [29]. Upregulation of the proline reductase operon in the transcriptome of mid-log cultures (**Table 1**) might also indicate that loss of *feoB1* in BHI media negatively impacted toxin production [11, 13]. Without Δ *feoB1*, *C. difficile* lacks *in vivo* fitness and could potentially be outcompeted by other gut flora that also compete for available iron. Indeed, the Feo system is also essential for other intestinal pathogens such as *E. coli*, *Helicobacter pylori* [32] and *Salmonella typhimurium* [15], which encode a single FeoB homolog. For example, without the Feo system *S. typhimurium* is unable to successfully colonize the large intestine of mice, but it can grow in blood and systemic body sites [15]. Similarly, *H. pylori* requires the FeoB system to colonize mice gastric mucosa though it encodes transporters for ferric iron and can acquire iron from host lactoferrin and transferrin [32, 33]. Our findings suggest that even if other forms of iron were available at the site of infection and/or *C. difficile* expressed other ferrous iron transporters (e.g., FeoB2 or ZupT), FeoB1 appears to be indispensable for colonization and pathogenesis. In a *C. difficile* CD630 *fur* mutant, iron starvation was shown to downregulate pili and flagella associated genes [16] that contribute to *C. difficile* adherence and colonization. We also observed downregulation of the Type IV pilin, surface layer protein A (*slpA*) and adhesin encoded by *cwp66* (**Table 1**). In *C. difficile*, Type IV pilin contributes to cell-cell attachment in biofilms and adherence to epithelial cells; it was reported that in strains CD630 and R20291, deletion of *pilB1* (CD630_35120 and CDR20291_3349) resulted in poorer colonization of mice colons and such mutants were

outcompeted by their WT parents [34]. Thus, the avirulence of R20291 Δ *feoB1* in mice may be multifactorial, possibly involving reductions of ferrous iron uptake, toxin production and colonization of the gut epithelia.

Antivirulent strategies to treat CDI have primarily focused on inhibiting TcdA and TcdB, with bezlotoxumab (monoclonal antibody to TcdB) being the only approved treatment [35]. However, blocking the biosynthesis of TcdA and TcdB without inhibiting *in vitro* growth is an attractive discovery-oriented approach for novel CDI therapeutics [36], which could complement existing treatment approaches [35]. Given the rapidly increasing understanding of *C. difficile* pathophysiology it may now be possible to target specific proteins and pathways that promote toxin production [37]. In this regard, FeoB1 which is central to *C. difficile* metabolism and pathogenesis could be a valuable drug target to discover novel CDI therapeutics.

MATERIALS AND METHODS

Strains and culture conditions. *C. difficile* R20291 and its isogenic *feoB1* deletion mutant (R20291 Δ *feoB1*) were routinely grown in pre-reduced BHI broth or agar at 37°C in a Whitley A35 anaerobic workstation (Don Whitley Scientific). *E. coli* SD46 was grown at 37°C in Luria–Bertani (LB) broth or agar. D-cycloserine (250 µg/ml), cefoxitin (8 µg/ml), and thiamphenicol (15 µg/ml) were used to selectively culture *C. difficile* containing plasmids, whereas chloramphenicol (15 µg/ml) and ampicillin (50 µg/ml) were used to grow *E. coli* SD46. Strains used are listed in **Table S2**.

Cell culture and conditions. Vero epithelial cells were cultured in 10% fetal bovine serum and Dulbecco's Modified Eagle Medium at 37°C with 5% carbon dioxide in a humidified incubator. These culture conditions were used in cell rounding assays.

Genetic manipulation of *C. difficile*. R20291 Δ *feoB1* was generated by allelic exchange, exactly as we described (19). Essentially, *E. coli* SD46 carrying the *feoB1* allelic deletion cassette in pMTL-SC7215 was conjugated into R20291, before subjecting the recombinant strain to serial passage to select for double crossover mutants. The deletion was confirmed by PCR and Sanger sequencing. R20291 Δ *feoB1* was complemented with WT *feoB1* expressed from its promoter in pMTL84151, as we described (19). The strain was maintained on pre-reduced BHI supplemented with thiamphenicol (15 µg/ml). The primers used in the study are listed in **Table S3**.

Quantification of *C. difficile* toxins. Strains were grown anaerobically in pre-reduced BHI at 37°C for 24 h. Samples were centrifuged at 5000 rpm for 5 min and supernatants recovered for ELISA and cell rounding assays, as follows. **(i) ELISA quantification.** About 100 µl of sample was used to measure TcdA and TcdB using the tgcBIOMICS GmbH ELISA kit that quantifies both toxins, against standard curves from purified TcdA and TcdB, according to the manufacturer's instructions. **(ii) Standard cell rounding assay.** Toxin titers were determined against Vero epithelial cells (~10⁵ cells/ml) that were adhered overnight. Supernatants from neat and various diluted samples were added to the cells and incubated for 4 h. Cytopathic effects (cell rounding) were visualized by microscopy, using six views per well. Toxin titers were defined as the lowest dilution in which cell rounding was no longer visualized. **(iii) Determination of cell rounding by morphometric analysis.** A similar approach was previously reported to quantify cell rounding in

a high throughput manner [22]. Briefly, 50 μ l of 500-1000 Vero cells were seeded per well of a 384-well black plate and incubated for ~18 h. To generate a standard curve purified TcdB (List Biological Laboratories, Inc) were diluted. From a source plate containing diluted TcdB and undiluted culture supernatants 0.05 μ l of samples were automatedly transferred to the 384-well plate containing the Vero cells using Echo® Acoustic Liquid Handler. This represented an *in situ* dilution of 1:1000 of the culture supernatants. Samples were incubated at 37°C for 6 h in 5% carbon dioxide, followed by addition of 10 μ l of 25% (v/v) paraformaldehyde. Cells were fixed overnight at 4°C, washed with PBS and stained with the nucleic acid stain DAPI (25 μ l). After incubation in the dark for 15 min at room temperature, they were stained with Alexa Fluor 488 phalloidin strain (40 μ l) at a concentration of 0.0006 μ M in PBS with 5% milk powder solution. After staining in the dark for 2 h, cells were washed twice, and PBS (50 μ l) was added. Cell images were captured using IN Cell Analyzer 6000 from GE Healthcare, with DAPI (excitation 358 nm and emission 451 nm) and phalloidin (excitation 495 nm and emission 518 nm). Using Pipeline Pilot version 9.2, automated morphometric analysis was done on: cell shape, coverage of the well surface area by cells, perimeter, and circularity. From these parameters the percentage of rounded cells were determined against the standard curve for TcdB.

Quantification of toxins in mice ceca. Cecal samples were collected and flash frozen. Samples (normalized to g/ml) were homogenized in PBS containing cOmplete™ Protease Inhibitor Cocktail from Roche, then centrifuged at 10, 000 rpm for 5 min. The supernatants (5 μ l) were used to quantify toxins by the above standard cell rounding assay.

RNA Sequencing and analysis. Triplicates of R20291 and R20291 Δ *feoBI* were grown overnight in pre-reduced BHI broth. The next day, cultures were diluted 1:100 in pre-reduced BHI broth and grown in the anaerobic chamber. Approximately 20 ml samples of each strain were harvested at three-time points OD~0.3, ~0.6, and ~0.9 and further aliquots of each time point also stored for subsequent analyses. These time points were selected to assess changes in gene transcription at different points in the bacterial growth cycle. Bacterial RNAprotect™ reagent (QIAGEN) was added and RNA isolated using Qiagen's RNeasy mini kit. RNA quality assessment, library preparation and Hi-Seq 2 x150 paired-end sequencing were performed by GENEWIZ (New Jersey, USA).

Analysis of RNA sequencing data. CLC Genomics Workbench version 12.0 was used to analyze RNA sequencing data. After trimming the adapters, the data was checked for its quality and relatedness using Principal Component Analysis (PCA). Further, *C. difficile* R20291 coding sequence (CDS, GenBank accession number: FN545816.1) was used for alignment and analysis. Differential gene expression of R20291 Δ *feoBI* was analyzed against R20291 wild type. The raw RNA-Seq data are deposited in NCBI database under accession number PRJNA753147.

Transcriptional analysis by RT-qPCR. RNA isolated from *C. difficile* samples was converted to cDNA by M-MLV reverse transcriptase (Quantabio) using qScript cDNA SuperMix. qScript One-Step SYBR Green qRT-PCR Kit, ROX (Quantabio) and gene-specific primers were used to amplify genes in Applied Biosystems ViiA7 real-time PCR machine. Transcript levels were calculated by the comparative Ct method ($\Delta\Delta$ CT method) and data normalized to 16S rRNA [38]. For analysis of cytokine levels in the cecal tissues, the same Qiagen's RNeasy mini kit was used

to isolate RNA and qRT-PCR was carried out as above. The data was normalized to the beta-actin gene.

Ethics statement

Experiments were conducted under animal use protocol # 2018-0054-IBT, approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Mouse model of colitis CDI. The model of CDI was modified from that described by Zhou et al. [23]. Male and female C57/BL6 mice (6 weeks) were from Envigo. For 5 days, mice were given water containing kanamycin (0.4 µg/ml), gentamicin (0.035 µg/ml), colistin (850 U/ml), metronidazole (0.215 µg/ml), vancomycin (0.045 µg/ml) and 1.5% w/v of dextran sodium sulfate. This was followed by two days of regular drug-free water. On day-1, mice were injected (i.p.) with clindamycin (10 mg/kg). Approximately 20 h later (day 0) they were infected with 10⁵ spores of either R20291 or R20291Δ*feoBI*. Three sets of experiments were carried out, as summarized in the results. *C. difficile* spores were prepared according to a standard protocol [39]. After infection, fecal samples were collected daily, and ceca recovered from euthanized moribund animals or at the experimental endpoint. Animals were monitored three times per day and scored for: health appearance, including hunched posture, ruffled fur, and diarrhea (wet tail); weight loss; and decreases in body temperature (**Table S4**). Moribund animals were compassionately sacrificed.

Analysis of cecal and fecal bioburdens. Samples were weighed, homogenized in PBS and normalized to g/ml. To enumerate spore burdens, samples were heated at 70°C for 30 minutes, diluted and then plated onto *C. difficile* selection agar (containing cycloserine, ceftiofur, and

fructose) supplemented with 0.1% w/v sodium taurocholate. After anaerobic incubation for up to 48 h viable counts were determined.

Quantification of intracellular iron by ICP-OES. Overnight grown cells were subcultured (1:100) in fresh BHI broth and harvested after 6 h for ICP-OES analysis to determine the cellular iron levels as we previously described [19].

Quantification of intracellular pyruvate. This assay was performed as we previously described [19]; essentially harvesting samples at OD~0.3, 0.6 and 0.9 to measuring intracellular pyruvate in cell lysates using the Pyruvate Colorimetric/Fluorometric Assay kit from Biovision.

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580 TABLES AND FIGURES

581 **Table 1.** Selected genes^a in *C. difficile* R20291 that were differentially expressed in

582 R20291 Δ *feoB1* compared to WT R20291.

Functional group/proteins	Gene	$\Delta feoB1$ vs WT (Log2(FC) ≥ 0.58)		
		Mid log	Late log	Stationary
Flavodoxin, iron metabolism and metal transport				
Flavodoxin	<i>fldx</i> (CDR20291_1925)	4.33	3.87	2.91
Riboflavin synthase beta-chain	<i>ribH</i> (CDR20291_1595)			1.34
Riboflavin synthase alpha chain	<i>ribB</i> (CDR20291_1597)	1.27	1.41	1.52
Riboflavin biosynthesis bifunctional diaminohydroxyphosphoribosylaminopyrimidine deaminase and 5-amino-6-(5- phosphoribosylamino)uracil reductase]	<i>ribD</i> (CDR20291_1598)	1.19	1.24	1.61
Bacterioferritin	<i>bfr</i> (CDR20291_2756)	-2.9	-2.57	-2.53
Ferrichrome- specific ABC transporter permease, ATP-binding protein	<i>fhuC</i> (CDR20291_2771)	0.92	0.73	
Ferrichrome- specific ABC transporter permease, substrate binding protein	<i>fhuD</i> (CDR20291_2774)	1.16		-1.52
Zinc Transporter	<i>zupT</i> (CDR20291_0946)	1.42	0.83	1.63
ABC-type Fe3+ transport system, permease component	<i>CDR20291_2980</i>	-7.61	-7.37	-7.91
ABC-type spermidine/putrescine transport systems, ATPase component	<i>CDR20291_2981</i>	-7.19	-7.55	-8.09
Iron (III) transport system substrate-binding protein	<i>CDR20291_2983</i>	-7.1	-7.51	-8.46

ABC-type Fe ³⁺ transport system, solute-binding protein	<i>CDR20291_3362</i>	2.83	2.62	2.38
ABC-type Fe ³⁺ transport system, permease component	<i>CDR20291_3364</i>	4.39	4.29	4.12
ABC-type Fe ³⁺ transport system, ATPase component (polyamine transport)	<i>CDR20291_3365</i>	4.11	4.59	3.7
Ferrous iron transporter subunit A2	<i>feoA2 (CDR20291_1367)</i>	3.06	3	1.81
Ferrous iron transporter subunit B2	<i>feoB2 (CDR20291_1366)</i>	2.05	1.91	1.90
Cobalt ABC transporter, ATP-binding protein	<i>cbiO (CDR20291_0332)</i>	0.91	1.51	
Polyamine biosynthesis				
S-adenosylmethionine decarboxylase	<i>speD (CDR20291_0819)</i>	-1.27	-0.96	-1.2
Spermidine synthase	<i>speE (CDR20291_0820)</i>	-0.69		
Energy metabolism				
Pyruvate carboxylase	<i>pyc (CDR20291_0010)</i>	-1.31	-1.4	-1.14
Pyruvate formate lyase	<i>pflD (CDR20291_3143)</i>		1.63	
Pyruvate-formate lyase-activating enzyme	<i>pflE (CDR20291_3144)</i>	0.87	1.76	
Homocitrate synthase NifV-type	<i>CDR20291_0762</i>	-3.11	-1.55	-3.69
Aconitase/3-isopropylmalate dehydratase	<i>CDR20291_0763</i>	-2.35	-2.47	-2.58
Isocitrate/3-isopropylmalate dehydrogenase	<i>CDR20291_0764</i>	-2.58	-1.89	-2.85
Succinyl-CoA:coenzyme A transferase	<i>catI (CDR20291_2232)</i>	6.19	6.55	5.38
Succinate-semialdehyde dehydrogenase [NAD(P)+]	<i>sucD (CDR20291_2231)</i>	6.39	6.74	5.48
Electron transfer flavoprotein alpha-subunit	<i>etfA2 (CDR20291_0912)</i>	1.64	2.42	1.91
Electron transfer flavoprotein beta-subunit	<i>etfB2 (CDR20291_0911)</i>		1.78	
Acetyl-CoA acetyltransferase	<i>thlA1 (CDR20291_0915)</i>		2.08	

3-hydroxybutyryl-CoA dehydratase	<i>crt2 (CDR20291_0913)</i>	1.14	2.11	1.53
Butyryl-CoA dehydrogenase complex	<i>bcd2 (CDR20291_0910)</i>		1.76	
Maltose phosphorylase	<i>mapA (CDR20291_2094)</i>	1.4	1.49	1.31
Maltose/glucose PTS system EIICB component	<i>CDR20291_2402</i>	1.8	1.86	1.95
Carbon catabolite repressor protein A	<i>ccpA (CDR20291_0923)</i>	0.77		

Proline reductase

Proline reductase subunit proprotein	<i>prdA (CDR20291_3103)</i>		0.57	
Proline reductase	<i>prdB (CDR20291_3101)</i>		0.65	
Putative electron transfer protein	<i>prdC (CDR20291_3105)</i>		0.54	
Proline racemase	<i>prdF (CDR20291_3097)</i>		0.69	

Pathogenicity locus (PaLoc)

Toxin A	<i>tcdA (CDR20291_0584)</i>	-6.64	-6.53	-9.47
Toxin B	<i>tcdB (CDR20291_0582)</i>	-6.14	-6.21	-8.94
Positive toxin regulator	<i>tcdR (CDR20291_0581)</i>	-4.51	-4.9	-6.15
Holin-like protein	<i>tcdE (CDR20291_0583)</i>	-5.63	-6.36	-7.83

Surface proteins and Pili

Cell surface protein	<i>cwp66 (CDR20291_2678)</i>		-0.59	
Surface layer protein A	<i>slpA (CDR20291_2682)</i>	-2.26	-2.1	-2.35
Type IV prepilin leader peptidase	<i>CDR20291_3340</i>	-0.78	-1.2	0.19
Type IV prepilin leader peptidase	<i>CDR20291_3341</i>	-0.96	-0.75	-1.01
Type IV pilus retraction protein	<i>CDR20291_3342</i>	-0.83	-0.65	
Type IV pilin	<i>CDR20291_3345</i>	-0.67	-0.78	
Type IV pilus assembly protein	<i>CDR20291_3346</i>	-0.64		
Type IV pilus assembly protein	<i>CDR20291_3348</i>	-0.73	-0.89	

Type IV pilus assembly protein	<i>CDR20291_3349</i>	-0.73
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583

584 ^aThe above genes were selected from **Table S1** for the following reasons: (i) Representation of
585 pathways affected by iron limitation in cells, including iron and metal acquisition transporters
586 and indicative of shift to flavodoxin metabolism (supported by riboflavin biosynthesis) and
587 energy metabolism pathways; (ii) indication that polyamine biosynthesis was not enhanced as a
588 means to acquire iron in BHI; and (iii) pathogenicity was affected in terms of expression of
589 PaLoc genes and surface proteins that help *C. difficile* to colonize the large bowel. Log2Fold
590 changes are based on $p \leq 0.02$.

Figure 1. Deletion of *feoB1* attenuates toxin production in *C. difficile* R20291. **A.** Loss of *feoB1* did not affect the growth of R20291. **B.** Without FeoB1, there was a ~ 25% reduction in intracellular iron content in R20291Δ*feoB1*. The amounts of toxins TcdA and TcdB were diminished in R20291Δ*feoB1* as determined by manual cell rounding analysis in **C**, showing toxin titers against Vero epithelial cells and in **D**, quantification of toxins by ELISA. Based on the more sensitive cell rounding assay, there was ~ 1000-fold less toxins produced by the mutant. Vero cells are more predictive for cell rounding caused by TcdB. **E.** These results were confirmed by automated morphometric analysis. Images show that more cell rounding was caused by the neat supernatant of WT R20291 than that of the mutant (1:1000 *in situ* dilution). In the untreated control, the yellow arrow shows the morphology of Vero cells, of actin filaments stained with phalloidin (green) and nuclei stained with DAPI (red); in cells treated with WT supernatants, the

yellow arrow shows the morphology of rounded cells. In cells treated with supernatants from the mutant, most cells appeared healthy. Statistical significance was determined by unpaired t-tests with Welch's correction in Graphpad Prism 9.0; asterisks correspond to p values of * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$; ns=not significant $p > 0.05$.

Figure 2. Deletion of FeoB1 attenuates colonization and virulence in mice. **A.** Schematic of the experimental timeline and procedure summary of the colitis CDI model used to evaluate the pathogenesis of R20291 and R20291 Δ feoB1. **B.** Ceca recovered from the mice infected with the wild type were red and inflamed in contrast to that from the *feoB1* mutant, which resembled the uninfected control mice ceca. **C.** Kaplan-Meier survival analysis revealed significant difference (p=0.0003) between mortality risks of mice infected with R20291 compared to R20291 Δ feoB1. **D.** Animals infected with R20291 exhibited significant weight loss (p=0.0009) compared to those infected with R20291 Δ feoB1. **E.** Endpoint cecal bioburdens revealed that deceased mice infected with R20291 had higher bacterial loads than mice that survived infection with R20291, while mice infected with the mutant R20291 Δ feoB1 had even lower bioburdens. Data above are based on group numbers of n=16 mice infected with R20291 and n=15 mice infected with R20291 Δ feoB1 (a mouse from the R20291 Δ feoB1 group was removed from the study, as it was inadvertently infected with R20291, as confirmed by PCR on cecal isolates). Statistical significance for Kaplan-

640 Meier survival were determined by log-rank test in Graphpad Prism 9.0. Results in E were
641 analyzed by one-way ANOVA with alpha 0.05 in Graphpad Prism 9.0. Other statistical analyses
642 were determined by unpaired t-tests with Welch's correction in Graphpad Prism 9.0.
643

Figure 3. Comparative analysis of virulence in mice at 48 h post-infection. **A.** Significant weight loss was seen 48 hours after infection with R20291 whereas mice infected with R20291 $\Delta feoB1$ gained weight, which was also significant. **B, C.** Reduced colonization by R20291 $\Delta feoB1$ was also evident from bioburdens analyzed from the ceca taken at 48 hours and fecal samples recovered at 24 and 48 h. **D, E.** Lower toxin titers and expression of cytokine in cecal tissues were also evident. Statistical significance was determined by unpaired t-tests with

Welch's correction in Graphpad Prism 9.0; asterisks correspond to p values of * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$; ns=not significant $p > 0.05$.

Figure 4. Transcriptome changes associated with lack of *feoB1* in R20291. **A.** Venn diagrams show total differentially expressed genes (DEGs) and those that were upregulated or downregulated at different growth phases (mid logarithmic OD600nm=0.3; late-logarithmic OD600nm=0.6; and stationary phase OD600nm=0.9). **B, C.** Gene Ontology analysis of DEGs

identify different metabolic processes that are likely altered by loss of *feoB1*, including iron homeostasis, pathogenesis, and redox reactions.

Figure 5. Effect of loss of FeoB1 on iron-responsive gene transcription, as determined by RT-qPCR. Reduced iron uptake triggered genes involved in iron metabolism; upregulated were flavodoxin (*fldx*), ferrichrome-specific ABC transporter permease (*fhuC* and *fhuD*), ferric uptake regulator (*fur*) and riboflavin biosynthesis genes (*ribB* and *ribD*), while bacterioferritin (*bfr*) was downregulated.

Figure 6. Relationship between *tcdA* expression and pyruvate accumulation in cells. **A.** RT-qPCR confirmed the downregulation of *tcdA*, *tcdB*, *tcdD* and *tcdE*, in R20291Δ*feoB1* late-logarithmic (OD600nm=0.6) and stationary phase cultures (OD600nm=0.9). **B.** Pyruvate accumulation was determined and shown to be higher in R20291Δ*feoB1* across the three growth phases. Intracellular concentrations of pyruvate in R20291 at each growth phase were: 1.16 ± 0.13 nm, 1.39 ± 0.08 nm and 1.66 ± 0.11 nm, respectively; intracellular concentrations of pyruvate in R20291Δ*feoB1* at each growth phase were 1.25 ± 0.14 nm, 1.55 ± 0.11 nm and 2.24 ± 0.16 nm. **C.** Downregulation of genes encoding enzymes of the oxidative branch of the Krebs cycle of *C. difficile*. This is consistent with reduced metabolism of pyruvate via that pathway; *pyc* encodes Pyruvate carboxylase; *CDR20291_0763* aconitase; and *CDR20291_0764* encodes Isocitrate/3-isopropylmalate dehydrogenase.

Figure 1

Figure 2

A

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B

C

D

E

Figure 3

A

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B

C

Figure 4

Stationary phase

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Late log phase

Mid log phase

Figure 5

A **B** **C**

Figure 6