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BACTERIAL INFECTIONS

Analysis of Six *tonB* Gene Homologs in *Bacteroides fragilis* Revealed That *tonB3* is Essential for Survival in Experimental Intestinal Colonization and Intra-Abdominal Infection

Anita C. Parker,[a] Nathaniel L. Seals,[a] Cecile L. Baccanale,[b] Edson R. Rocha[a]

[a]Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, North Carolina, USA
[b]Department of Comparative Medicine, Brody School of Medicine, East Carolina University, Greenville, North Carolina, USA

ABSTRACT The opportunistic, anaerobic pathogen and commensal of the human large intestinal tract, *Bacteroides fragilis* strain 638R, contains six predicted TonB proteins, termed TonB1-6, four ExbBs orthologs, ExbB1-4, and five ExbDs orthologs, ExbD1-5. The inner membrane TonB/ExbB/ExbD complex harvests energy from the proton motive force (Δp), and the TonB C-terminal domain interacts with and transduces energy to outer membrane TonB-dependent transporters (TBDTs). However, TonB's role in activating nearly one hundred TBDTs for nutrient acquisition in *B. fragilis* during intestinal colonization and extraintestinal infection has not been established. In this study, we show that growth was abolished in the Δ*tonB3* mutant when heme, vitamin B_{12}, Fe(III)-ferrichrome, starch, mucin-glycans, or N-linked glycans were used as a substrate for growth *in vitro*. Genetic complementation of the Δ*tonB3* mutant with the *tonB3* gene restored growth on these substrates. The Δ*tonB1*, Δ*tonB2*, Δ*tonB4,* Δ*tonB5,* and Δ*tonB6* single mutants did not show a growth defect. This indicates that there was no functional compensation for the lack of TonB3, and it demonstrates that TonB3, alone, drives the TBDTs involved in the transport of essential nutrients. The Δ*tonB3* mutant had a severe growth defect in a mouse model of intestinal colonization compared to the parent strain. This intestinal growth defect was enhanced in the Δ*tonB3* Δ*tonB6* double mutant strain, which completely lost its ability to colonize the mouse intestinal tract compared to the parent strain. The Δ*tonB1*, Δ*tonB2*, Δ*tonB4,* and Δ*tonB5* mutants did not significantly affect intestinal colonization. Moreover, the survival of the Δ*tonB3* mutant strain was completely eradicated in a rat model of intra-abdominal infection. Taken together, these findings show that TonB3 was essential for survival *in vivo*. The genetic organization of *tonB1*, *tonB2*, *tonB4, tonB5,* and *tonB6* gene orthologs indicates that they may interact with periplasmic and nonreceptor outer membrane proteins, but the physiological relevance of this has not been defined. Because anaerobic fermentation metabolism yields a lower Δp than aerobic respiration and *B. fragilis* has a reduced redox state in its periplasmic space—in contrast to an oxidative environment in aerobes—it remains to be determined if the diverse system of TonB/ExbB/ExbD orthologs encoded by *B. fragilis* have an increased sensitivity to PMF (relative to aerobic bacteria) to allow for the harvesting of energy under anaerobic conditions.

KEYWORDS B. fragilis, Bacteroides, TonB, TonB-dependent transporter, anaerobic bacteria, intestinal colonization, intra-abdominal infection

The TonB dependent outer membrane transporters (TBDT), also known as ligand-gated porins, are involved in the transport of several nutrients such as ferric iron bound-siderophores, vitamin B_{12}, heme, metals (cobalt, copper, and nickel), and oligosaccharides across the outer membrane (1–3). Many TBDTs are multifunctional as they participate in bacteriophage adsorption and entry, and as receptors for bacteriocins (4, 5). The TBDTs are energized by the periplasmic C-terminal domain of TonB following its interaction with a short

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Address correspondence to Edson R. Rocha, rochae@ecu.edu.

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amino acid sequence at the N- terminal region (TonB box) of TBDTs. Energy is harvested from the transmembrane proton motive force (Δp) at the cytoplasmic membrane by the TonB/ExbB/ExbD complex and is transduced by TonB to the TBDTs (5–8).

In organisms such as the commensal *Escherichia coli* K12, the TonB/ExbB/ExbD system is required to energize seven TBDTs (six are dedicated to the uptake and transport of ferric iron bound-siderophores and one to vitamin B_{12} uptake), while clinical isolates of *E. coli* and other Gram-negative bacterial pathogens contain 8 to 22 TBDTs for the uptake of ferric iron-siderophores, heme, or other metal complexes (3, 6). The TonB/ExbB/ExbD-dependent energy transduction mechanisms are well studied in aerobic and facultatively anaerobic bacteria. These systems are typically for the acquisition of insoluble ferric iron as it is the dominant iron valence in oxygenated environments. In contrast, under anaerobic conditions or at low pH, free ferrous iron is more abundant and several pathways for ferrous iron uptake have been described (9). Studies in *E. coli* have shown that the *tonB* gene is repressed anaerobically under iron-replete conditions, but it is highly expressed anaerobically, under iron-limiting conditions, at levels even higher than aerobic conditions (10). The uptake of Fe^{3+}-siderophore chelates has been shown to occur in anaerobic environments as well (11). In addition, the siderophore enterobactin is also synthesized anaerobically (12), and several TBDTs involved in ferric iron-bound siderophore uptake are also upregulated and produced anaerobically (11, 13). This suggests that the TonB/ExbB/ExbD energy transduction mechanism is functional regardless of oxygenation conditions. This might be an important physiological feature for nutrient acquisition by intestinal Gram-negative bacteria, which are transiently mobilized to oxygenated and non-oxygenated niches, localized at proximal mucosal barriers or far into the lumen of the intestinal tract, respectively (14–16).

Homologs of the TonB/ExbB/ExbD system are also widespread in anaerobic bacteria such as the *Bacteroides* species that colonize the human lower intestinal tract. In the genomes of relevant clinical and commensal strains of *Bacteroides* species isolated from human specimens, *tonB* gene homologs are found to be present in several copies ranging from 6 in *B. fragilis*, 8 in *B. ovatus*, to 11 in *B. thetaiotaomicron* ([1, 17–20], https://www.ncbi.nlm.nih.gov/genome/?term=Bacteroides). However, there is a paucity of information regarding the specific requirements of TonB, and its interactions with TBDTs anaerobically. It is assumed that multiple TonB proteins may be required for interactions with subsets of TBDTs (21, 22). However, the contributions of TonB/ExbB/ExbD orthologs in the stable colonization of the lower intestinal tract and during opportunistic extraintestinal infection by anaerobic bacteria are not well understood.

In organisms containing multiple homologs of the TonB protein, there seem to be specific requirements for each TonB to interact with subsets of TBDTs for the transport of chelated iron and other nutrients, and with other bacterial physiological activities whose mechanisms are yet to be defined (23–25). In *Bradyrhizobium japonicum*, the heme TBDT HmuR is TonB1-dependent while the outer membrane xenosiderophore transporters FhuE, FegA, and EntR, which are utilized for the import of Fe^{3+}-chelates of desferrioxamine, ferrichrome, and enterobactin, respectively, are TonB2-dependent (26). In *Vibrio* species, TonB1 and TonB2 are not fully redundant as TonB1 is associated with heme and ferrichrome transport, while TonB2 is essential for the transport of anguibactin, vanchrobactin, enterobactin and ferrichrome (27). In *Vibrio cholerae*, heme is transported through the HasR outer membrane receptor, which can also be energized with the TonB2 system, while HutA and HutR, which transport heme, function with either TonB1 or TonB2 but preferentially associate with TonB1 (28). In *Aeromonas hydrophila*, only the TonB2 system is required to utilize iron from iron-binding proteins and plays a role in adhesion, motility, and biofilm formation while TonB3 is involved in anti-phagocytic properties. Interestingly, the *A. hydrophila tonB*123 triple mutant has increased susceptibility to erythromycin and roxithromycin (29). Although *Sphingobium* sp. strain SYK-6 contains six TonB homologues, only TonB1 was essential for the outer membrane DdvT transporter to uptake the aromatic compound derived from lignin degradation 5,5′-dehydrodivanillate (30). *Acinetobacter baumannii* TonB1 and TonB2 are significantly deficient in growth under iron restricted conditions, while TonB3 is essential for bacterial viability but independent of iron content conditions (31). Inactivation of *tonB1* in

Pseudomonas aeruginosa inhibits both iron-bound siderophore and heme uptake, but inactivation of the *tonB2* gene has no effect on iron or heme acquisition, although a *tonB1 tonB2* double mutant has a stronger growth defect in iron restricted conditions than the *tonB1* single mutant alone. Additionally, the *tonB3* mutant is defective in twitching motility and assembly of type IV pili components (23). In this regard, pathogenic bacteria require TonB-dependent active transport for nutrient assimilation during infection, and the mutation of the *tonB* gene attenuates virulence and survival in experimental *in vivo* models of infection (32–37).

The requirements for distinct TonB proteins in *Bacteroides* spp. have not been defined, and little information is available on the importance of TonB in the pathophysiology of the opportunistic human pathogen *B. fragilis*. The *Bacteroides* species contain a large number of TBDTs varying approximately from 100 to 120 needed for the transport of a vast number of nutrients including dietary plant and animal polysaccharides, host glycans, vitamin B_{12}, heme, and iron-bound xenosiderophores, but for many of these TBDTs receptors, the specific substrates and nutritional roles remain unknown (21, 22, 38–43). Therefore, it remains to be defined whether *Bacteroides* TonBs have specific requirements that are related to their ability to interact with TBDTs and/or ExbBs/ExbDs that are essential for survival in host tissues. In this study we have used the commensal and opportunistic pathogen *B. fragilis* as a model to understand the requirement of TonB for nutrient assimilation *in vitro* and for the ability of this organism to survive in experimental intestinal colonization and extraintestinal infection.

RESULTS

Genetic organization of *B. fragilis* 638R *tonB* genes. The *B. fragilis* 638R chromosomal organizations of the six *tonB*, four *exbB* and five *exbD* gene homologs are shown in Fig. 1A The *tonB4* gene is adjacent to the *exbD3* and *exbB3* genes together with a gene encoding for a pyridoxine biosynthesis (PdxJ) protein. The *tonB6*, *exbB4*, *exbD4*, and *ExbD5* genes are clustered together with genes encoding a periplasmic binding protein type 2, and a protein with tetratricopeptide repeats (TPR). The *tonB1*, *tonB2*, *tonB3*, and *tonB5* genes are not encoded adjacent to *exbB* or *exbD* genes. The *exbD1*, *exbD2*, *exbB1*, and *exbB2* genes are organized together in the same transcriptional orientation.

Comparison of amino acid sequences. A BLAST search of the *B. fragilis* 638R genome using the *E. coli* TonB protein C-terminal domain region as a search query revealed the presence of six putative proteins containing amino acid sequence homology to the C-terminal domain of the *E. coli* TonB and to other Gram-negative bacterial TonBs (Fig. 1B). The signature *E. coli* TonB "YP" motif Y163 and P164 (24) is conserved among the bacterial TonB family of proteins and is preserved in all *B. fragilis* 638R TonBs except for TonB2 and TonB4, where the Y residue is substituted with H324 and L216, respectively. In addition, the Gly174, Val176, and Gly 186 (*E. coli* TonB) are conserved in all six *B. fragilis* TonBs. The V225 (*E. coli* TonB) is also conserved except that it is replaced by I325 in TonB2. The W213 and F230 (*E. coli* TonB) are conserved in *B. fragilis* TonBs except that W213 (*E. coli*) is replaced by A258 in TonB4.

The amino acid sequence alignment of *B. fragilis* 638R TonB orthologs with other *Bacteroides* species' putative TonB proteins revealed that *B. thetaiotaomicron* VPI-5482 contains 11 putative TonBs; *Phocaeicola* (*Bacteroides*) *vulgatus* ATCC8482 contains 6 putative TonBs; and *Bacteroides ovatus* ATCC 8483 contains 8 putative TonBs (Fig. S1). These *Bacteroides* TonBs contain the signature "YP" motif and the conserved Gly174, Val176, and Gly 186 from *E. coli* TonB. The *P. vulgatus* TonB1 (BVU_0265_TonB1) was included in the alignment because the YP motif and the Gly174, Val176, and Gly 186 residues (*E. coli* TonB) are conserved in the second C-terminal TonB domain found in long TonBs that contain the MecR1/BlaR1-like transmembrane domain (24). BVU_2559 (*P. vulgatus* putative TonB) lacks the "YP" residues, although it is included in the alignment as it contains the Gly174, Val176, and Gly 186 (*E. coli* TonB) conserved amino acid residues. The alignment revealed that the C-terminal amino acid regions I125 to G128, N149 to K151, K184 to V198, and R217 to T219 (referred to BF638R_3560_TonB3) are conserved or have conserved substitutions in $>$50% of *Bacteroides* species TonBs with no significant homology to TonBs from other aerobic bacteria (Fig. S1). All the predicted TonB proteins from *B. ovatus* ATCC 8483 and *B. thetaiotaomicron* VPI 5482 contain the characteristic "YP" C-terminal signature residues.

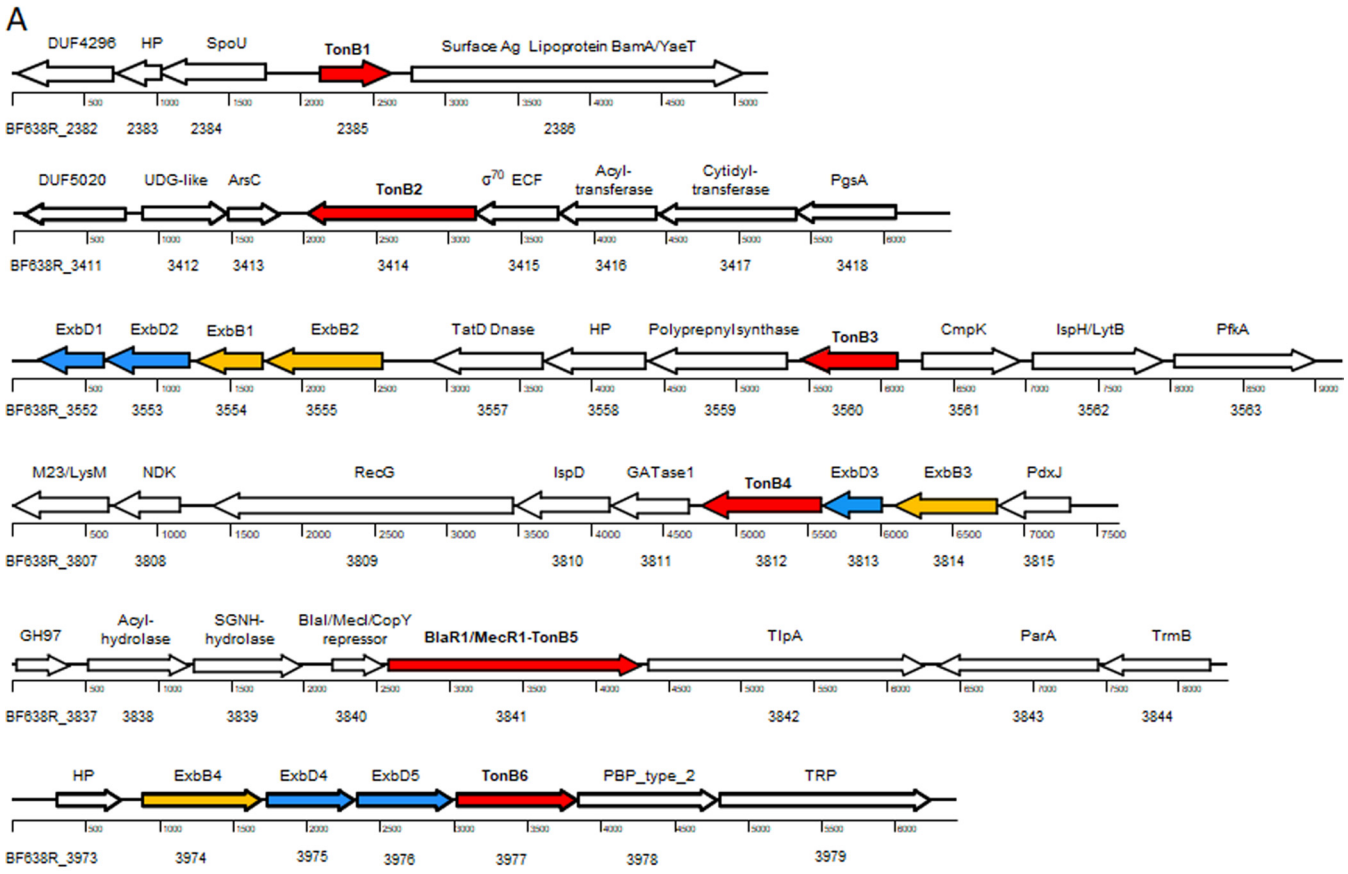

FIG 1 (A) Schematic representation of the *B. fragilis 638R* chromosomal region of the six *tonB* gene orthologs. Each tag locus is depicted below the respective putative ORF symbolized by an arrow. The thin dark line with numbers placed at intervals represents the scaled length of the DNA fragment in bp. Arrows filled with color represent the functional annotation group assigned to TonB (red), ExbB (yellow), or ExbD (blue) orthologs, respectively. The designation of the predicted peptide product is depicted above each open arrow gene region respectively. Arrow direction depicts the transcription orientation. DUF4296: putative lipoprotein containing a domain of unknown function. HP: conserved hypothetical protein. SpoU: RNA methyltransferase superfamily. DUF5020: putative exported protein containing a domain of unknown function. UDG: uracil DNA glycosidase family. ArsC: arsenate reductase family. σ^{70} ECF: RNA polymerase sigma factor, sigma-70 family. PgsA: Phosphatidylglycerophosphate synthase. CmpK: CMP kinase. IspH/LytB: putative isoprene biosynthesis related reductase. PfkA: 6-phosphofructokinase. M23/LysM: Peptidase_M23 and LysM domain-containing protein superfamily. NDK: Nucleoside diphosphate kinase. RecG: ATP-dependent DNA helicase. IspD: d-ribitol-5-phosphate cytidylyltransferase. GATase1: Type 1 glutamine amidotransferase (GATase1)-like domain. PdxJ: Pyridoxal phosphate biosynthesis protein. GH97: Glycosyl-hydrolase family 97. SGNH hydrolase: an exported

(Continued on next page)

The N-terminal domains showed no significant homology (Fig. S1) as expected for the TonB superfamily of proteins (24). A bioinformatic analysis revealed that the N-terminal domains of *B. fragilis* 638R TonB2, TonB3, TonB4, and TonB6 contain one transmembrane helix domain. TonB5 has a long N-terminal domain containing four transmembrane domains with homology to the MecR1/BlaR1 superfamily of proteins as described for other *B. fragilis* strains (24). BF638R_2383_TonB1 contains N-terminal sequence residues that overlap a transmembrane domain and a predicted export signal peptide (Table S2 and Fig. S1). The TonB N-terminal transmembrane helical domains showed poor homology among *Bacteroides* species, although conserved substitutions of hydrophobic amino acids comprise a majority of the consensus residues (Fig. S2). The number of predicted transmembrane helices, signal peptide export sequences, or prokaryotic cleavage site motifs for lipid attachment in the N-terminal domain of TonB orthologs of *B. ovatus* ATCC 8483, *B. thetaiotaomicron* VPI 5382, and *P. vulgatus* ATCC 8482 are shown in Table S2 and Fig. S1. The significance of the lack of N-terminal transmembrane domain and the presence of a signal peptide to the function of TonB homologs was not further analyzed in this study.

The effect of *tonB* deletion mutants on nutrient utilization *in vitro*. Henceforth, to investigate the function of *B. fragilis* TonB homologs, in-frame *tonB* deletion mutants were created. The *tonB* mutant strains were tested to determine if they would abolish the utilization of different substrates that are known to be transported by TBDTs in *B. fragilis* (41, 44–47). To test nutrient utilization, bacteria were grown on a modified defined medium containing one substrate to be assimilated by an outer membrane TonB-dependent transporter as described in the text.

When the six Δ*tonB* mutant strains were grown on starch or mucin-glycans as a carbon source, the Δ*tonB3* mutant (BER-190) growth was completely abolished compared to the parent strain (Fig. 2A and C). In addition, the Δ*tonB1* Δ*tonB2* Δ*tonB3* triple mutant (BER-213), Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* quadruple mutant (BER-214), and Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6* sextuple mutant (BER-196) strains failed to grow on starch or mucin glycans compared to parent strain (Fig. S3). No growth defect was observed with Δ*tonB1* (BER-188), Δ*tonB2* (BER-189), Δ*tonB4* (BER-191), Δ*tonB5* (BER-192), and Δ*tonB6* (BER-193) strains (Fig. 2A and C), nor with the Δ*tonB1* Δ*tonB2* double mutant (BER-194), Δ*tonB5* Δ*tonB6* double mutant (BER-262), or Δ*tonB4* Δ*tonB5* Δ*tonB6* triple mutant (BER-195) strains (Fig. S3). Genetic complementation with the native *tonB3* gene in the Δ*tonB3* *tonB3*$^+$ (BER-197), Δ*tonB1* Δ*tonB2* Δ*tonB3* *tonB3*$^+$ (BER-264), and Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6* *tonB3*$^+$ (BER-265) strains completely restored growth on starch and mucin-glycans (Fig. 2B and D; Fig. S3).

The bacterial growth on Fe(III)-ferrichrome as a sole source of iron was strongly reduced in the Δ*tonB3* strain compared to parent strain (Fig. 3A). No significant growth inhibition was seen for the Δ*tonB1,* Δ*tonB2,* Δ*tonB4,* Δ*tonB5,* and Δ*tonB6* strains. Growth of the Δ*tonB3* deletion mutant on protoporphyrin IX plus ferrous ammonium sulfate was used as control (Fig. 3B). Genetic complementation of the Δ*tonB3* mutant strain with the *tonB3* gene restored growth around the paper disk impregnated with Fe(III)-ferrichrome on medium containing protoporphyrin IX plus the ferrous iron chelator bathophenanthroline disulfonic acid (Fig. 3C).

When the bacterial strains were grown on defined media with heme (Fig. 4A), vitamin B_{12} (Fig. 4B), rabbit serum (Fig. 4D), rat serum (Fig. 4E), or the human serum N-linked glycoprotein, transferrin (Fig. 4F), the Δ*tonB3* mutant failed to grow on any of these substrates compared to the parent strain. In contrast, no significant growth defect was seen with the

FIG 1 Legend (Continued)
protein belonging to the diverse family of lipases and esterases. TlpA: exported disulfide reductase protein. ParA: Fe-S cluster assembling factor nucleotide binding protein. TrmB: tRNA methyltransferase. PBP_type_2: periplasmic binding protein Type 2 superfamily. TRP: exported tetratricopeptide repeat lipoprotein. (B) Multiple alignment of the amino acid sequences from the C-terminal domains of six *B. fragilis* 638R TonB protein homologs with other bacterial TonB protein C-terminal domains from *E. coli* (BAA14784), *P. aeruginosa* (NP_254218), *V. cholera* TonB1 (NP_233295) and TonB2 (KUP88502), and *Y. pestis* (NP_405736). The conserved amino acid residues (>50%) are labeled with black boxes. Conserved amino acid substitutions are depicted by gray boxes. Alignment of the protein sequences was performed using Clustal Omega algorithm from the MegAlign Pro component of the DNASTAR Lasergene software Version 17.2.1 (DNASTAR, Inc., Madison, WI). Asterisks below the sequence depict 100% amino acid conservation, and dots below the sequence depict >50% conserved amino acid substitutions. Shading of the multi-sequence alignment ".msf" file was performed using the Boxshade webserver program at https://embnet.vital-it.ch/software/BOX_form.html. The amino acid position numbering is shown for each aligned sequence, respectively.

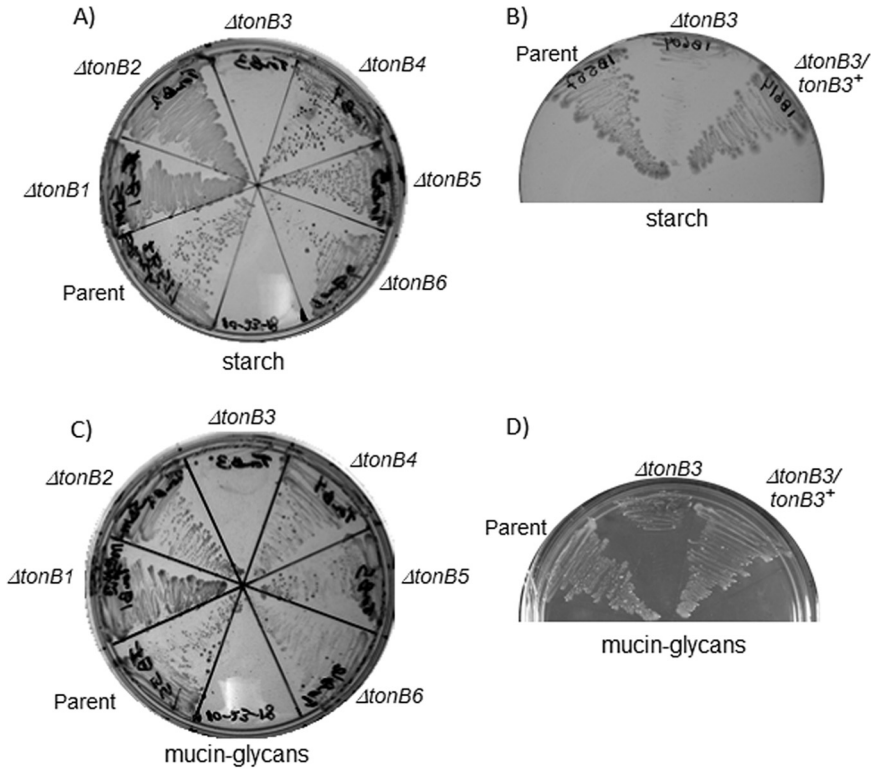

FIG 2 Growth of *B. fragilis* 638R *tonB* deletion mutant strains on modified defined media containing starch (A and B) or mucin-glycans (C and D). Defined medium without d-glucose was supplemented with soluble starch (0.5%) or mucin-glycan extract (1.5%). Hemin was omitted and replaced with protoporphyrin IX (5 μg/mL) plus ferrous ammonium sulfate (100 μM), and vitamin B_{12} was omitted and replaced with L-methionine (75 μg/mL) as described in Materials and Methods. Strain designations are depicted for each panel. Parent: *B. fragilis* 638R Δ*tdk* (BER-183). Δ*tonB1* (BER-188), Δ*tonB2* (BER-189), Δ*tonB3* (BER-190), Δ*tonB4* (BER-191), Δ*tonB5* (BER-192), Δ*tonB6* (BER-193). (B and D) Genetic complementation assays for the Δ*tonB3 tonB3*$^+$ (BER-197) strain.

Δ*tonB1* (BER-188), Δ*tonB2* (BER-189), Δ*tonB4* (BER-191), Δ*tonB5* (BER-192), or Δ*tonB6* (BER-193) mutant strains compared to parent strain (Fig. 4). The growth of Δ*tonB3* strain on defined medium glucose containing protoporphyrin IX plus ferrous iron and L-methionine was used as control (Fig. 4C). The Δ*tonB1* mutant strain consistently had a growth rate higher than the

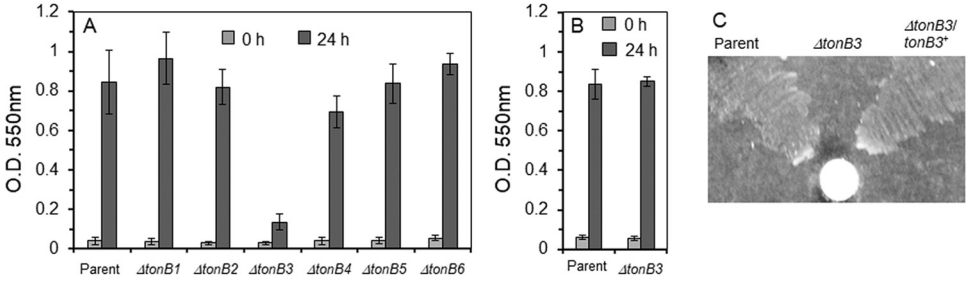

FIG 3 Growth of *B. fragilis* 638R *tonB* deletion mutant strains on Fe(III)-ferrichrome. (A) Defined media glucose containing 5 μg/mL protoporphyrin IX and 20 μM bathophenanthroline disulfonic acid (BPS) as a ferrous iron chelator for exogenous free-iron-limiting conditions. Hemin was omitted and L-methionine replaced vitamin B_{12}. Fe(III)-ferrichrome (5 μM final concentration) was added to the media as described previously (46). (B) Defined medium glucose containing 5 μg/mL protoporphyrin IX plus 100 μM ammonium ferrous sulfate was used as control. (C) Genetic complementation assay for the Δ*tonB3 tonB3*$^+$ (BER-197) strain. Fe(III)-ferrichrome solution (0.5 mM) was added to the paper disk on BHIS plate containing 5 μg/mL protoporphyrin IX plus 1 mM BPS as described previously (46). Strain designations are labeled in each panel. Parent: *B. fragilis* 638R Δ*tdk* (BER-183). Δ*tonB1* (BER-188), Δ*tonB2* (BER-189), Δ*tonB3* (BER-190), Δ*tonB4* (BER-191), Δ*tonB5* (BER-192), and Δ*tonB6* (BER-193). Vertical bars denote standard deviation of the means from two independent experiments in triplicate.

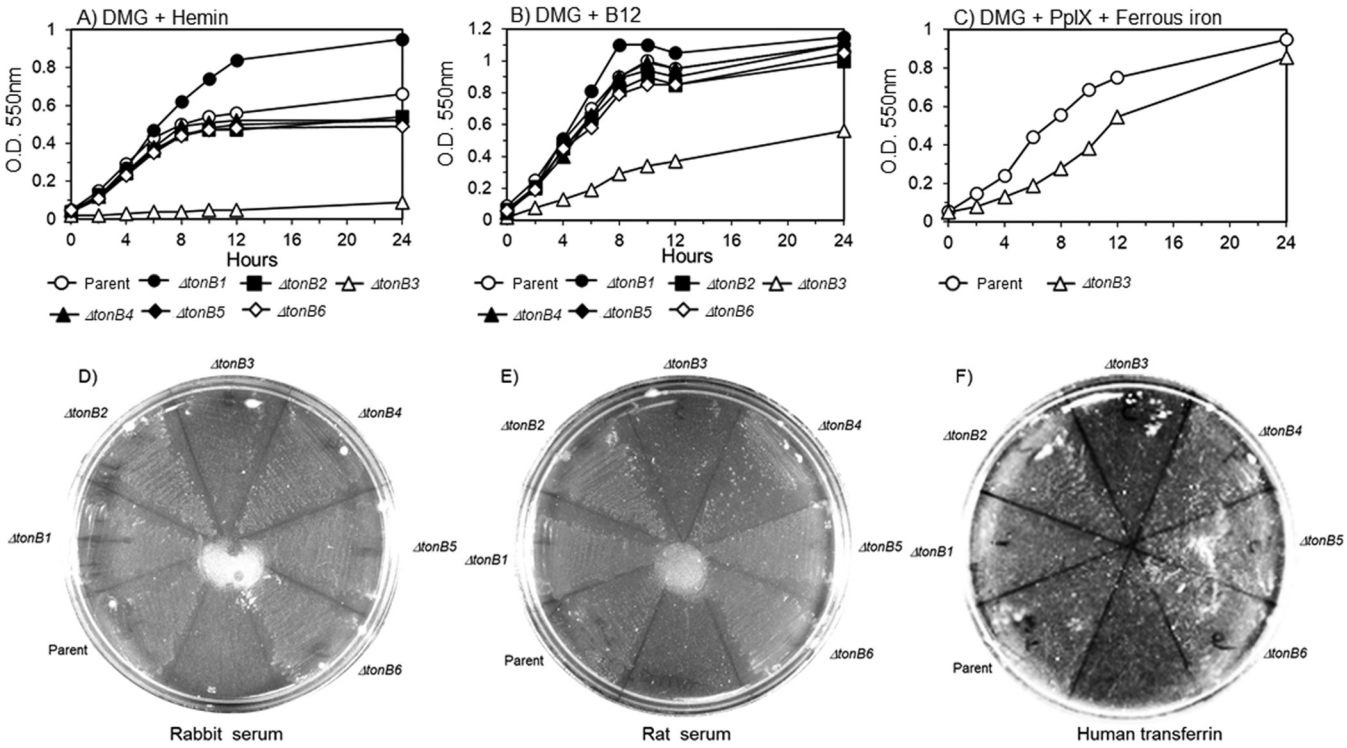

FIG 4 Growth of *B. fragilis* 638R *tonB* deletion mutant strains on different substrates. (A–C) The defined medium glucose (DMG) was prepared as described in the Materials and Methods section with the following modifications. (A) Hemin was added at 5 μg/mL and bathophenanthroline disulfonic acid was added at 400 μM to obtain exogenous free-iron-limiting conditions. Protoporphyrin IX and ferrous ammonium sulfate were omitted. Vitamin B12 was omitted and replaced with L-methionine (75 μg/mL). (B) Vitamin B12 was added at 5 μg/liter, L-methionine was omitted. Hemin was omitted and replaced with protoporphyrin IX at 5 μg/mL and 100 μM ammonium ferrous sulfate. C) Defined media containing protoporphyrin IX (5 μg/mL), ammonium ferrous sulfate (100 μM), and L-methionine (75 μg/mL) were used as control. (D–F) The defined medium without glucose was supplemented with (D) rabbit serum (20%), (E) rat serum (20%), or (F) human apotransferrin (20 mg/mL). Heme and vitamin B_{12} were replaced with protoporphyrin and L-methionine, respectively. Symbols and strain designations are labeled in each panel. Parent: *B. fragilis* 638R Δ*tdk* (BER-183). Δ*tonB1* (BER-188), Δ*tonB2* (BER-189), Δ*tonB3* (BER-190), Δ*tonB4* (BER-191), Δ*tonB5* (BER-192), and Δ*tonB6* (BER-193). Data presented are the average of one determination in duplicate.

parent strain in the presence of heme under inorganic iron limiting conditions (Fig. 4A). In addition, the Δ*tonB1* Δ*tonB2* Δ*tonB3* triple mutant (BER-213) and Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6* sextuple mutant (BER-196) did not grow on media containing the bovine serum glycoprotein, fetuin, compared to parent strain (Fig. S4). However, genetic complementation with *tonB3* gene completely restored growth of the Δ*tonB1* Δ*tonB2* Δ*tonB3* *tonB3*+ (BER-264), and Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6* *tonB3*+ (BER-265) strains on fetuin (Fig. S4).

Taken together, these findings show that TonB3 (but not TonB1, TonB2, TonB4, TonB5, or TonB6) is a functional TonB required for the energization of TBDTs involved in the transport of starch, glycans from host glycoproteins, and essential nutrients such as heme and vitamin B_{12}. Therefore, to demonstrate whether or not the *tonB* gene orthologs would have a significant contribution to pathophysiology and survival in a host environment *in vivo*, experimental intestinal colonization and intra-abdominal infection studies were carried out.

Growth of *tonB* deletion mutants in a mouse model of intestinal colonization. Dual colonization of the mouse intestinal tract with the parent strain and the Δ*tonB3* mutant strain (BER-190) showed a decrease of approximately 4 logs-fold in the Δ*tonB3* mutant population compared to the parent strain after 7 days of colonization (Fig. 5G). The mean CFU/g of cecal content of the Δ*tonB1* Δ*tonB2* Δ*tonB3* triple mutant (BER-213) was approximately 5 logs lower than the parent (Fig. 5B). The Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* quadruple mutant (BER-214) population was not significantly different from the Δ*tonB1* Δ*tonB2* Δ*tonB3* triple mutant (Fig. 5C). The Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* quintuple mutant (BER-215) population decreased approximately 6 logs-fold compared to the parent strain (Fig. 5D). However, the Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6* sextuple mutant (BER-196) population was completely outcompeted by the parent

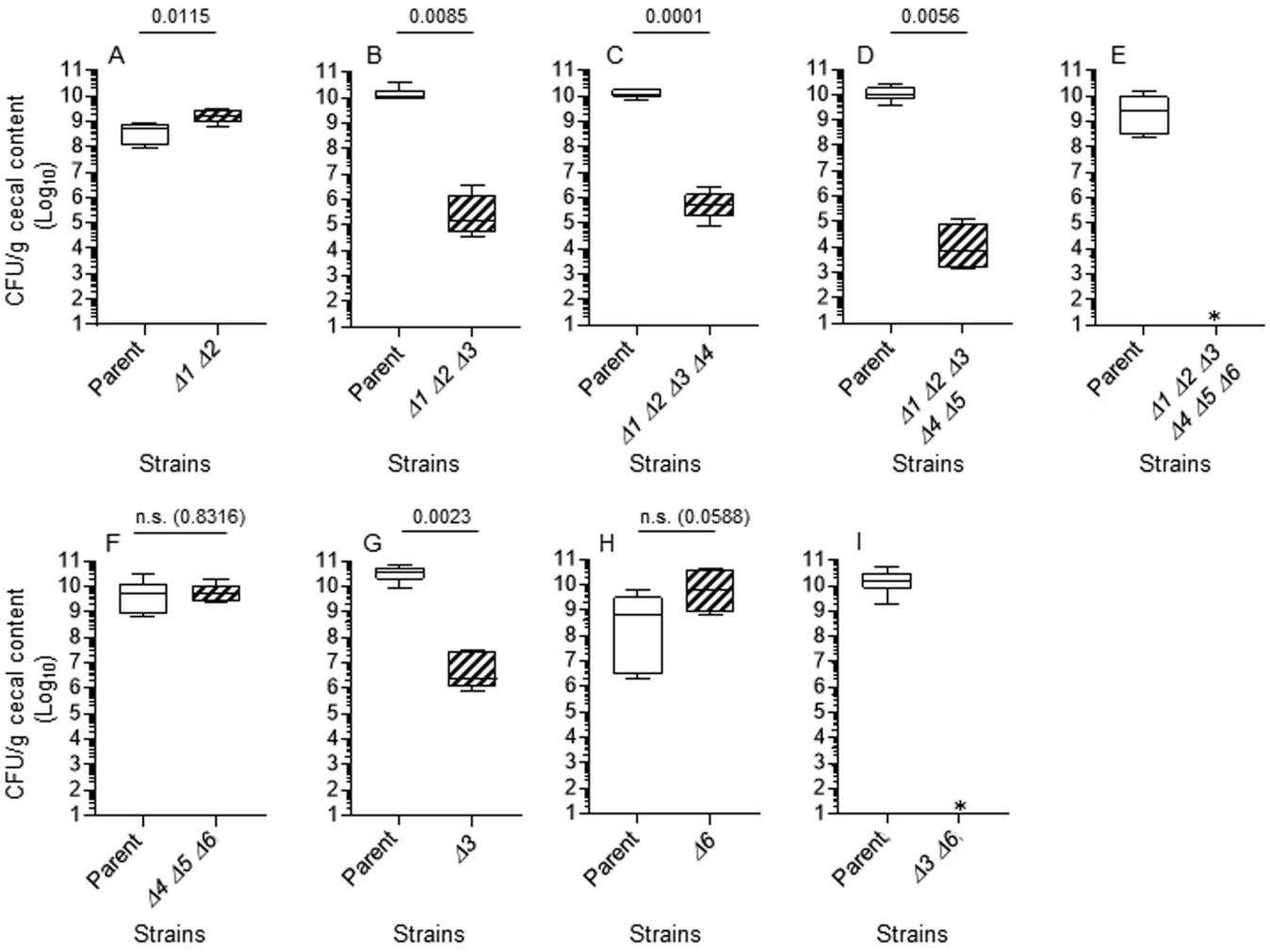

FIG 5 Competitive dual-bacterial colonization of C57BL/6J mouse intestinal tract with *B. fragilis* strains. (A) parent-Erm^r (BER-208) versus Δ*tonB1* Δ*tonB2*-Tet^r (Δ*1* Δ*2*), BER-230. (B) parent-Erm^r (BER-208) versus Δ*tonB1* Δ*tonB2* Δ*tonB3*-Tet^r (Δ*1* Δ*2* Δ*3*), BER-220. (C) parent-Erm^r (BER-208) versus Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4*-Tet^r (Δ*1* Δ*2* Δ*3* Δ*4*), (BER-221). (D) parent-Erm^r (BER-208) versus Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5*-Tet^r (Δ*1* Δ*2* Δ*3* Δ*4* Δ*5*), BER-222. (E) parent-Erm^r (BER-208) versus Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6*-Tet^r (Δ*1* Δ*2* Δ*3* Δ*4* Δ*5* Δ*6*), BER-211. (F) parent-Erm^r (BER-208) versus Δ*tonB4* Δ*tonB5* Δ*tonB6*-Tet^r (Δ*4* Δ*5* Δ*6*), BER-219. (G) parent-Erm^r (BER-208) versus Δ*tonB3*-Tet^r (Δ*3*), BER-209. (H) parent-Erm^r (BER-208) versus Δ*tonB6*-Tet^r (Δ*3*), BER-224. (I) parent-Erm^r (BER-208) versus Δ*tonB3* Δ*tonB6*-Tet^r (Δ*3* Δ*6*), BER-228. Erm^r: erythromycin resistance marker in strains carrying pNBU2-*bla-ermG*, Tet^r: tetracycline resistance marker in strains carrying pNBU2-*bla-tetQ*. Two and 4 days after stopping antibiotic treatment, mice (n = 6) were inoculated with the dual bacterial suspension at a 1:1 ratio in 0.2 mL PBS containing an average of 1.05×10^{10} to 2.0×10^{10} CFU/mL for each stain by oral gavage as described in Materials and Methods. After 7 days, the cecal content was serially diluted and plated on BHIS containing the appropriate antibiotics for selection and enumeration of colonies. Colony counts were normalized to CFU/g of cecal content. Mice were placed on the standard rodent chow diet. The whiskers denote the minimum and maximum values. The line in the middle of the box is plotted at the median. The boxes span the interquartile range. The significance of the P value following an unpaired t test (parametric and two-tailed) of the two groups is shown above the horizontal bars. n.s., not significant. Panels (E) and (I) were not analyzed for statistical significance. Asterisks depict no detectable CFU counts (below detection limit of 1×10^1 CFU/mL).

strain (Fig. 5E). There was no detectable survival (below 1×10^1 CFU/g detection limit) in the cecal content compared to the parent strain. This indicates that there might be a strong synergistic effect between TonB3 and TonB6 with regard to enabling optimal intestinal colonization. This synergistic growth defect was confirmed by demonstrating that the survival of the Δ*tonB3* Δ*tonB6* double mutant strain (BER-227) was completely abolished compared to parent strain in a dual-strain competitive intestinal colonization (Fig. 5I). Interestingly, the Δ*tonB6* single mutant (BER-193) had no competitive growth defect compared to parent strain (Fig. 5H). Both the Δ*tonB1* Δ*tonB2* double mutant (BER-194) and the Δ*tonB4* Δ*tonB5* Δ*tonB6* triple mutant (BER-195) strains did not show significant differences in the CFU counts compared to the parent strain, respectively (Fig. 5A and F).

When single strains were inoculated in the mouse intestinal tract, the BER-190 strain (Δ*tonB3* mutant) also showed a growth defect in the noncompetitive colonization

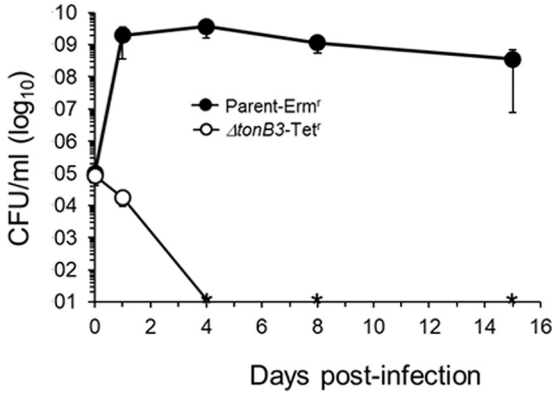

FIG 6 Competitive survival of *B. fragilis* strains inoculated into the rat intraperitoneal tissue cage. Dual infection with parent-Erm^r (BER-208) versus Δ*tonB3*-Tet^r (BER-209) strains. Bacteria grown overnight in BHIS medium were diluted in PBS and mixed at a 1:1 ratio to approximately 1×10^5 CFU/mL of each strain. Four milliliters of the dual bacterial suspension were inoculated into the intraperitoneal tissue cage. Fluid samples were aspirated at time points for CFU counts as described in Materials and Methods. Data are expressed as the mean CFU per milliliter of intra-abdominal tissue cage fluid from six rats. The standard errors of the means (SEM) are denoted by vertical error bars. Asterisks depict the time points postinfection with no detectable CFU counts (below detection limit of 1×10^1 CFU/mL).

assay (Fig. S5). The Δ*tonB3* mutant had an approximately 2 logs-fold decrease in CFU counts in the mouse cecum compared to the parent strain. The genetic complementation of the Δ*tonB3* mutant strains (BER-197) completely restored the CFU counts to parent strain level. The BER-194 (Δ*tonB1* Δ*tonB2*) and the BER-195-(Δ*tonB4* Δ*tonB5* Δ*tonB6*) strains did not show a significant growth defect compared to the parent strain. Taken together, these results show that TonB3 plays an important role in the ability of *B. fragilis* to colonize the intestinal tract.

The *tonB3* gene is essential for *B. fragilis* growth in an experimental model of intra-abdominal infection. Previous studies have shown that *B. fragilis* has an essential requirement for heme and N-linked-glycans to survive in extra-intestinal infections (44, 48). Since the Δ*tonB3* mutant failed to grow on heme and N-linked glycoproteins in *in vitro* assays, the Δ*tonB3* mutant (BER-190) was tested for survival in extra-intestinal tissues. When equal numbers of parent and Δ*tonB3* mutant cells were used to coinfect intra-abdominal rat tissue cages, the surviving population of the mutant strain was rapidly outcompeted by the surviving parent strain. The Δ*tonB3* mutant strain was unable to grow in the intra-abdominal tissue cage compared to the parent strain (Fig. 6). Its CFU/mL counts had decreased by nearly 1 log-fold by day 1 postinfection compared to its initial CFU inoculum counts. The Δ*tonB3* cells were completely eradicated (below detection level of 1×10^1 CFU/mL)) after day 4 postinfection and remained undetectable until day 15 postinfection (Fig. 6). Necropsies were performed at the end of these experiments and no indication of an inflammatory process or presence of abscesses was observed outside the implanted tissue cage. Therefore, no leakage of the content of the tissue cage occurred and there was no bacterial dissemination to the tissues and organs of the peritoneal cavity for either the parent or Δ*tonB3* mutant strains. The other Δ*tonB* mutant strains were not tested for intra-abdominal survival in this study.

DISCUSSION

In this study we show that *B. fragilis* contains six *tonB* gene homologs, and the deletion of the *tonB3* gene completely abolished the ability of *B. fragilis* 638R to survive in extra-intestinal infection. However, in the intestinal tract, mutating *tonB3* and *tonB6* together abolishes intestinal colonization. This synergistic effect demonstrates that TonB6 has some role in the environmental conditions of the intestinal tract, although it is not observed *in vitro*. In *in vitro* assays, the *tonB3* gene was essential for growth in the presence of heme, vitamin B_{12}, iron-bound-ferrichrome, soluble starch, mucin-derived glycans, and serum N-linked glycoproteins. Taken together, these findings showed that there is no apparent redundancy or overlapping function

among *B. fragilis* TonB homologs, as far as the TonB3 requirement for the utilization of the substrates tested is concerned.

Overall, very little is known about the role of TonB protein homologs and their interactions with TBDTs in *B. fragilis*. *B. fragilis* contains 104 predicted TBDTs, which are grouped in two distinct phylogenetic clades: 33 are predicted to contain the 22-strand antiparallel β-barrel signature of siderophore transporters (46), and 71 belong to the family of SusC-like nutrient transporters (44). Although we do not know the full extent of TBDT subsets associated with TonB3, we predict that TonB3 interacts and provides energy transduction for BF638R_3439 (DonC), a SusC-like transporter required for the utilization of N-linked glycans (44), and to BF638R_3170 (OsuA), a SusC-like transporter of starch/maltooligosaccharides (45). This assumption is based on the fact that the *donC* and *osuA* mutants, like Δ*tonB3*, do not assimilate N-glycans or starch, respectively (44, 45). In addition, HmuR homologs of the siderophore receptor family (BF638R_2501 and BF638R_2717), involved in heme acquisition (41), are also likely to be linked to TonB3. Moreover, the fact that the mucin-glycan extract was not utilized by the Δ*tonB3* mutant as carbon source indicates that the TBDT(s) needed for assimilation of O-linked-glycans also interact with TonB3. These findings strengthen our evidence that TonB3 is the major energy transducer for TBDTs involved in the assimilation of host-derived glycans, and in the assimilation of the essential *B. fragilis* nutrients heme and vitamin B_{12}.

The molecular interactions of TonB3 with TBDTs have not yet been experimentally established, but a consensus sequence of eight amino acids (LDEVVVvG) at the N-terminal plug region of 85 TBDTs, including DonC (BF638R_3439) and OsuA (BF638R_3170) transporters (Fig. S6), share strong homology to the TonB box QVVVLG (Q103 to G108) of *P. gingivalis* RagA (49), to the TonB box IDEVVVT (residues 23–29) of *B. thetaiotaomicron* OMP121, BT_2264 (50), and to the *V. cholera* HutA (DEVVVST) and HutR (EEVVV) TonB boxes (51). If this putative "TonB3 box" is correct, there might be other features, in addition to the TonB3 box, that allow the TonB3 to interact with its target transporter as neither TonB1, TonB2, TonB4, TonB5, nor TonB6 were able to compensate for the lack of TonB3. One of these features is the interaction of TonB C-terminal region with the ExbD periplasmic domain to adopt a correct conformation to bind to its target. (7, 52, 53). Therefore, we posit that there exists a coordinated control that directs the required ExbB1, ExbB2, ExbB3, or ExbB4 subunits and ExbD1, ExbD2, ExbD3, ExbD4, or ExbD5 subunits to assemble a subunit complex with the correct combination of ExbB(s) and ExbD(s) for the energization of TonB3. In *E. coli*, the ExbBD complex is composed of a homohexamer or homopentamer of ExbB monomer subunits and a homotrimer or homodimer of ExbD monomers enclosed around a central channel for harvesting PMF and subsequent energization of the TonB via a currently undefined mechanism (8, 54). Thus, the mechanism behind the formation of the ExbB/ExbD subunit complex to specifically recruit and energize TonB3 to attain its correct conformational structure to energize and activate its target remains to be defined.

Another aspect of TonB/ExbB/ExbD systems that has received little attention is the fact that during anaerobic fermentation, the Δp generated across the inner membrane is significantly lower than the Δp across the cytoplasmic membrane of aerobic organisms. Studies have shown that bacteria have a significant decrease in Δp during anaerobic fermentative catabolism compared to aerobic and anaerobic respiration (55–60). Taken from *E. coli* studies, it is expected that the vast number of TBDTs encoded by *B. fragilis* would outnumber the TonB proteins in the cell, leading to a competition of occupied receptors for energized TonB, resulting in cycles of binding and release of TBDTs known as an energization cycle (7, 61, 62). However, there is a paucity of information regarding the mechanisms that couple TonB/ExbB/ExbD to PMF in a reductive redox environment (63). Therefore, it remains to be determined if the anaerobic energy harvesting mechanisms necessary to trigger a vast number of ligand transport in anaerobic bacteria is comparable to the energy level for activation of aerobic bacterial transporters.

Taken from a previous study on genome expression of *B. fragilis* 638R grown on defined medium with glucose or mucin-glycans and in intra-abdominal tissue cage infection, microarray data showed that all the *tonB* gene orthologs are transcribed at levels similar to the

tonB3 transcriptional level ([44]; Fig. S7). Moreover, in the intestinal tract of gnotobiotic mice fed with a high-iron or a low-iron customized diet, the *tonB3* gene transcriptional level was higher than the other *tonB* gene orthologs (Fig. S7). These findings indicate that the *tonB1*, *tonB2*, *tonB4*, *tonB3*, *tonB5*, and *tonB6* genes are expressed in the nutrient assimilation and *in vivo* survival conditions used in this study. In fact, this suggests that the *tonB* orthologs may exert roles that have not been explored. Based on the structural genomic organization of *tonB1*, *tonB2*, *tonB4*, *tonB5*, and *tonB6*, it is possible that they are involved in providing the transduction of energy to periplasmic and OM energy-dependent functions other than to active transporters. For, example, the *tonB1* gene is localized immediately upstream of a surface antigenic lipoprotein (BF638R_2386) belonging to the BamA/TamA/YaeT superfamily of cell wall/membrane envelope biogenesis proteins that participate in the formation and assembly of complexes of a large number of proteins with β-barrel structures (like TBDTs) into the outer membrane without an obvious source of energy (64–66). In addition, the mechanisms that provide energy for the formation of outer membrane vesicles (OMV) in prokaryotes are not well defined (67–69). Since TonB can interact with nonreceptor proteins (70) and there is an absence of ATP and NADPH in the periplasmic space (67, 68, 71, 72), it will be interesting to see if *B. fragilis* TonB orthologs with structure-function dissimilar from the canonical *E. coli* TonB, could utilize TonB for the energization of β-barrel protein assembly and/or OMV segregation and partition and segmentation of the OM for shedding of OMV (67). The latter is a well-established phenomenon in *B. fragilis* associated with virulence, immunomodulatory function, and host–microbe communications in this organism (73–75).

In this regard, it is of relevance to mention that a putative exported thioredoxin-like protein, TlpA, belonging to the disulfide reductase TlpA, ResA, and DsbE protein superfamily, is encoded adjacent to the *tonB5* gene. TonB5 in *B. fragilis* belongs to a class of TonBs with a much longer N-terminal domain that has four transmembrane domains with high homology to BlaR1 and MecR1 proteins in *Staphylococcus aureus* involved in antibiotic resistance (24). Interestingly, a BlaI/MecI repressor homolog of the penicillinase-R superfamily is also encoded upstream of the *tonB5-tlpA* genes. Thus, it remains to be defined whether the unusual TonB5 structure would affect thiol-disulfide equilibrium in the reduced redox state of the *B. fragilis* periplasm (46, 76–80).

This study highlights the central role that TonB3 plays in *B. fragilis* pathogenesis. It also emphasizes the presence of non-canonical TonB orthologs whose structure-function has yet to be explored. In view of the abundance of discrete TBDTs in *Bacteroides* species (38, 81, 82), it points to the adaptation of TonBs to drive energy to TBDTs under anaerobic conditions. The assimilation of essential nutrients such as N-linked glycans and heme has been shown to be critical in *B. fragilis* extra-intestinal infections (44, 48, 83). Thus, the exploration of compounds that target nutrient assimilation metabolism and mechanisms of energy flux across membranes and the periplasm (84–86) might serve as potential targets for the development of novel antimicrobial therapeutics for the treatment of *B. fragilis* abscesses that are difficult to resolve due to increases in antibiotic resistance.

MATERIALS AND METHODS

Strains, media, and growth conditions. *B. fragilis* strains and plasmids used in this study are listed in Table 1. The BER-183 strain (*B. fragilis* 638R Δ*tdk*), used as the parent strain for the construction of isogenic mutants, was routinely grown on BHIS medium (brain heart infusion supplemented with L-cysteine (1 g/liter), hemin (5 mg/liter), and $NaHCO_3$ (20 mL of a 10% solution per liter) or otherwise stated in the text). Rifampin (20 μg/mL), gentamicin (100 μg/mL), erythromycin (10 μg/mL), tetracycline (5 μg/mL), and 5-fluor-2′-deoxyuridine, FUdR, (200 μg/mL) were added to the media when required. For the routine growth and maintenance of the *tonB* mutant strains, d-glucose (0.3%), yeast extract (0.5%), protoporphyrin IX (5 μg/mL), and 100 μM ferrous ammonium sulfate were added to the BHIS medium described above (reinforced-BHIS). The defined medium (87) was formulated as follows: KH_2PO_4 (1.15 g/liter); NH_4SO_4 (0.4 g/liter); NaCl (0.9 g/liter); L-methionine (75 mg/liter); $MgCl_2.6H_2O$ (20 mg/liter); $CaCl_2.2H_2O$ (6.6 mg/liter); $MnCl_2.4H_2O$ (1 mg/liter); $CoCl_2.6H_2O$ (1 mg/liter); resazurin (1 mg/liter); L-cysteine (1 g/liter); hemin (5 mg/liter); and d-glucose (5 g/liter). Final pH was 6.9. Vitamin B12 (5 μg/liter) was added when required. Twenty mL of sterile 10% $NaHCO_3$ were added per liter of medium inside the anaerobic chamber.

To test the effect of Δ*tonB* mutation on the transport of a specific nutrient, the defined medium described above was modified so that only one nutrient that is expected to be transported by a TonB-dependent transporter is available in the culture medium. This was performed because *B. fragilis* has an essential requirement for both heme and vitamin B_{12} (88), which require TBDTs for their assimilation. However, in the absence of heme, *B. fragilis* can synthesize heme if protoporphyrin IX and a source of inorganic iron are provided

TABLE 1 Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Description or genotype	Reference
Strains		
B. fragilis		
BER-183	*B. fragilis* 638R Δ*tdk*, Rif^r FUdR^r	(48)
BER-188	BER-183 Δ*tonB1*, Rif^r FUdR^r	This study
BER-189	BER-183 Δ*tonB2*, Rif^r FUdR^r	This study
BER-190	BER-183 Δ*tonB3*, Rif^r FUdR^r	This study
BER-191	BER-183 Δ*tonB4*, Rif^r FUdR^r	This study
BER-192	BER-183 Δ*tonB5*, Rif^r FUdR^r	This study
BER-193	BER-183 Δ*tonB6*, Rif^r FUdR^r	This study
BER-194	BER-183 Δ*tonB1* Δ*tonB2* Rif^r FUdR^r	This study
BER-195	BER-183 Δ*tonB4* Δ*tonB5* Δ*tonB6*, Rif^r FUdR^r	This study
BER-196	BER-183 Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6*, Rif^r FUdR^r	This study
BER-197	BER-183 Δ*tonB3 tonB3*$^+$ Rif^r FUdR^r Erm^r	This study
BER-208	BER-183 p*NBU2-bla- ermGb*, Rif^r FUdR^r Erm^r	(48)
BER-209	BER-190 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-211	BER-196 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-213	BER-183 Δ*tonB1* Δ*tonB2* Δ*tonB3*, Rif^r FUdR^r	This study
BER-214	BER-183 Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Rif^r FUdR^r	This study
BER-215	BER-183 Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Rif^r FUdR^r	This study
BER-219	BER-195 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-220	BER-213 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-221	BER-214 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-222	BER-215 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-224	BER-193 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-227	BER-183 Δ*tonB3* Δ*tonB6*, Rif^r FUdR^r	This study
BER-228	BER-227 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-230	BER-194 p*NBU2-bla-tetQb* Rif^r FUdR^r Tet^r	This study
BER-262	BER-183 Δ*tonB5* Δ*tonB6* Rif^r FUdR^r	This study
BER-264	BER-183 Δ*tonB1* Δ*tonB2* Δ*tonB3 tonB3*$^+$, Rif^r FUdR^r Erm^r	This study
BER-265	BER-183 Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6 tonB3*$^+$ Rif^r FUdR^r Erm^r	This study
E. coli		
DH10B	cloning host strain	Invitrogen
S17-1 λpir	Strain with the RK2 *tra* genes for conjugative transfer integrated in the chromosome (*RP4-2-Tc::Mu-Km::Tn7, pro, res*$^-$*mod*$^+$, Tp^r Sm^r) *λpir* lysogen	(92)
Plasmids		
pExchange-*tdk*	Derivative of pKNOCK-*bla-ermGb* carrying cloned *tdk* gene for counter-selection. (Amp^r) Erm^r	(93)
pNBU2-*bla-ermGb*	NBU2 integrase (*intN2*) based genomic insertion vector derived from pKNOCK-*bla-ermGb* inserts into NBU2 *att1* or *att2* sites of tRNA^{ser}. (Amp^r) Erm^r	(93)
pNBU2-*bla-tetQb*	NBU2 integrase (*intN2*) based genomic insertion vector derived from pKNOCK-*bla-tetQb* inserts into NBU2 *att1* or *att2* sites of tRNA^{ser}. (Amp^r) Tet^r	(81)
pFD1264	A 3,258 bp DNA fragment containing 601 bp in-frame deletion of *tonB6* gene BF638R_3977 was cloned into the XbaI/PstI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1266	A 3,922 bp DNA fragment containing 318 bp in-frame deletion of *tonB1* gene BF638R_2385 was cloned into the XbaI/SalI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1267	A 3,949 bp DNA fragment containing 995 bp in-frame deletion of *tonB2* gene BF638R_3414 was cloned into the XbaI/PstI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1268	A 1,905 bp DNA fragment containing 528 bp in-frame deletion of *tonB3* gene BF638R_3560 was cloned into the XbaI/PstI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1269	A 3,180 bp DNA fragment containing 692 bp in-frame deletion of *tonB4* gene BF638R_3812 was cloned into the XbaI/PstI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1270	A 3,237 bp DNA fragment containing 1,440 bp in-frame deletion of *tonB5* gene BF638R_3841 was cloned into the XbaI/PstI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1271	A 965 bp DNA fragment from *B. fragilis* 638R chromosome containing the native *tonB3* operon was cloned into the XbaI/PstI sites of pNBU2-*bla-ermGb* and used for genetic complementation of Δ*tonB3*. (Amp^r) Erm^r	This study

[a]Erm^r, erythromycin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance; Sp^r, spectinomycin resistance; FUdR^r, 5-fluor-2′-deoxyuridine resistance; Km^r, kanamycin resistance. Parentheses indicate antibiotic resistance expression in *E. coli*.

(89, 90), and vitamin B_{12} can be replaced with L-methionine (88). Therefore, when required, heme was replaced with protoporphyrin IX plus ferrous ammonium sulfate, and vitamin B_{12} was replaced with L-methionine to avoid the presence of multiple substrates that require TBDT for assimilation in the media altogether. This allowed us to determine if the assimilation of a single essential nutrient substrate could be affected by *tonB* mutation as measured by a growth defect phenotype. For complex polysaccharide assimilation, D-glucose was omitted and replaced with either soluble starch (0.5%), mucin-glycan (1.5%), rabbit serum (20%), rat serum

(20%), or human apotransferrin (20 mg/mL). Both heme and vitamin B_{12} were also omitted to avoid conflation of growth deficiency phenotypes; however, their replacements were added to enable growth as described above. For growth on Fe(III)-ferrichrome as a sole source of iron, the media were prepared exactly as described previously (46). The mucin glycan extract, which contains mostly O-linked but also some N-linked glycans, was prepared as described previously (44, 81).

Construction of in-frame null deletion mutations in the *tonB* genes. N-terminal and C-terminal DNA fragments flanking an internal DNA region to be deleted in-frame for each *tonB* gene, were amplified from the *B. fragilis* 638R chromosome by PCR using primers sets described in Table S1. The amplified DNA fragments were inserted into the cloning sites of the pExchange-*bla-ermGb* plasmid, respectively (Table 1). Each construct was mobilized from *E. coli* S17-1 λpir into BER-183 (BF638R Δ*tdk*) by biparental mating as described previously (48). Transconjugants were selected on reinforced BHIS containing rifampin (20 μg/mL), gentamicin (100 μg/mL), and erythromycin (10 μg/mL). Four colonies were picked up and grown overnight in 5 mL of reinforced-BHIS broth containing rifampin (20 μg/mL) and gentamicin (100 μg/mL). The Δ*tonB* enriched cultures were plated out on reinforced BHIS containing rifampin (20 μg/mL), gentamicin (100 μg/mL), and FUdR (200 μg/mL) as previously described (48). Colonies were tested for erythromycin sensitivity to confirm loss of the suicide vector. The FUdR resistant and erythromycin sensitive strains were selected for PCR analysis using TonB-mutcheck-F and TonB-mutcheck-R primers, respectively, to confirm deletion of an internal DNA fragment of the *tonB1* gene in BER-188 (BER-183 Δ*tonB1*), the *tonB2* gene in BER-189 (BER-183 Δ*tonB2*), the *tonB3* gene in BER-190 (BER-183 Δ*tonB3*), the *tonB4* gene in BER-191 (BER-183 Δ*tonB4*), the *tonB5* gene in BER-192 (BER-183 Δ*tonB5*), and the *tonB6* gene in BER-193 (BER-183 Δ*tonB6*) strains (Table 1).

Construction of Δ*tonB1* Δ*tonB2 double mutant. The plasmid pFD1267 carrying the Δ*tonB2* construct was mobilized from *E. coli* S17-1 λpir into BER-188 (Δ*tonB1*) stain by biparental mating. Transconjugants were selected on reinforced BHIS as described above. The FUdR resistant and erythromycin sensitive strains were selected for PCR analysis using TonB2-mutcheck-F and TonB2-mutcheck-R primers to confirm deletion of a 995 bp internal DNA fragment of the *tonB2* gene in the BER-194 (Δ*tonB1* Δ*tonB2*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-194 to introduce a tetracycline resistance marker into the BER-230 strain.

Construction of Δ*tonB4* Δ*tonB5* Δ*tonB6* triple mutant. The plasmid pFD1264 carrying Δ*tonB6* construct was mobilized from *E. coli* S17-1 λpir into BER-192 (Δ*tonB5*) strain by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive colonies were selected as previously described and analyzed by PCR using primers TonB6-mutcheck-F and TonB6-mutcheck-R to confirm deletion of the 601 bp internal DNA fragment of the *tonB6* in new Δ*tonB5* Δ*tonB6* strain. Then, the pFD1269 carrying ΔtonB4 was mobilized from *E. coli* S17-1 λpir into the Δ*tonB5* Δ*tonB6* double mutant strain by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive colonies were selected as previously defined and analyzed by PCR using primers TonB4-mutcheck-F and TonB4-mutcheck-R to confirm deletion of the 693 bp internal DNA fragment of the *tonB4* gene in the BER-195 (Δ*tonB4* Δ*tonB5* Δ*tonB6*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-195 to introduce a tetracycline resistance marker into the BER-219 strain.

Construction of Δ*tonB1* Δ*tonB2* Δ*tonB3* triple mutant. The plasmid pFD1268 carrying Δ*tonB3* construct was mobilized from *E. coli* S17-1 λpir into BER-194 (Δ*tonB1* Δ*tonB2*) by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive strains were selected for PCR analysis using primers TonB3-mutcheck-F and TonB3-mutcheck-R to confirm deletion of the 528 bp internal DNA fragment of the *tonB3* gene in the BER-213 (*tonB1* Δ*tonB2* Δ*tonB3*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-213 to introduce a tetracycline resistance marker into the BER-220 strain.

Construction of Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* quadruple mutant. The plasmid pFD1269 carrying Δ*tonB4* construct was mobilized from *E. coli* S17-1 λpir into BER-213 (Δ*tonB1* Δ*tonB2* Δ*tonB3*) by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive strains were selected for PCR analysis using primers TonB4-mutcheck-F and TonB4-mutcheck-R to confirm deletion of the 693 bp internal DNA fragment of the *tonB4* gene in the BER-214 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-214 to introduce a tetracycline resistance marker into the BER-221 strain.

Construction of Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* quintuple mutant. The plasmid pFD1270 carrying Δ*tonB5* construct was mobilized from *E. coli* S17-1 λpir into BER-214 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4*) by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive strains were selected for PCR analysis using primers TonB5-mutcheck-F and TonB5-mutcheck-R to confirm deletion of the 1,440 bp internal DNA fragment of the *tonB5* gene in the BER-215 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-215 to introduce a tetracycline resistance marker into the BER-222 strain.

Construction of Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6* sextuple mutant. The plasmid pFD1264 carrying Δ*tonB6* construct was mobilized from *E. coli* S17-1 λpir into BER-215 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5*) strain by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive colonies were selected as previously defined and analyzed by PCR using primers TonB6-mutcheck-F and TonB6-mutcheck-R to confirm deletion of the 601 bp internal DNA fragment of the *tonB6* gene in the BER-196 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-196 to introduce a tetracycline resistance marker into the BER-211 strain.

Construction of Δ*tonB3* Δ*tonB6* double mutant. The plasmid pFD1268 carrying Δ*tonB3* construct was mobilized from *E. coli* S17-1 λpir into BER-193 (Δ*tonB6*) strain by biparental mating. Transconjugants were selected as described above. The FUdR resistant and erythromycin sensitive colonies were selected as previously described and analyzed by PCR using primers TonB3-mutcheck-F and TonB3-mutcheck-R to confirm deletion of the 528 bp internal DNA fragment of the *tonB3* gene in the BER-227 (Δ*tonB3* Δ*tonB6*) strain. The plasmid pNBU2-*bla-tetQb* was mobilized into BER-227 to introduce a tetracycline resistance marker into the BER-228 strain.

Genetic complementation of Δ*tonB3*. A 965 bp DNA fragment containing the *tonB3* gene including 215 bp upstream of the start codon, ATG, containing promoter region and 49 bp downstream of the stop codon, was amplified by PCR from the *B. fragilis* 638R chromosome using primers TonB3Compl_XbaI-F and TonB3Compl_PstI-R. The amplified native *tonB3* operon was cloned into the XbaI/PstI sites of pNBU2-*bla-ermGb* plasmid. The new plasmid pFD1271 was mobilized from *E. coli* S17-1 λpir into BER-190 (Δ*tonB3*), BER-213 (Δ*tonB1* Δ*tonB2* Δ*tonB3*), and BER-196 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6*) to construct BER-197 (Δ*tonB3 tonB3*$^+$), BER-264 (Δ*tonB1* Δ*tonB2* Δ*tonB3 tonB3*$^+$), and BER-265 (Δ*tonB1* Δ*tonB2* Δ*tonB3* Δ*tonB4* Δ*tonB5* Δ*tonB6 tonB3*$^+$), respectively.

***In vivo* dual-bacterial competitive survival assays.** All procedures involving animals followed the guidelines given by the National Research Council's *Guide for the Care and Use of Laboratory Animals* (91) and approved by the Institutional Animal Care and Use Committee of East Carolina University.

(i) Intestinal colonization. Dual bacterial intestinal colonization of specific pathogen-free, 6–8-week-old male C57BL/6J mice purchased from Jackson Laboratories and housed under microisolation conditions (animal biological safety level-2 housing facility) was performed exactly as described previously (48). Mice were given drinking water with the antibiotics gentamicin (0.30 mg/mL), ciprofloxacin (0.66 mg/mL), and metronidazole (0.40 mg/mL). The sweetener Stevia extract (20 mg/mL) was added in the water to increase consumption. Antibiotic treatment was carried out for 7 days and subsequently replaced with sterile drinking water. Two days and 4 days following withdrawal of antibiotics, an inoculum containing a 1:1 ratio of the parent strain to the mutant strain was mixed to a total of approximately 2×10^9 CFU for each strain in 0.2 mL PBS and was given by oral gavage to 3 mice per group in two independent experiments ($n = 6$). Mice were housed with sterile autoclaved water and sterile irradiated standard rodent chow *ad libitum*. After 7 days, animals were euthanized. The cecum was slit open and cecal content specimens were weighed, serially diluted in sterile PBS, and plated on rifampin (20 μg/mL), gentamicin (100 μg/mL), and FUdR (200 μg/mL) plus erythromycin (10 μg/mL) or tetracycline (5 μg/mL) as described previously (48).

(ii) Intra-abdominal infection. Dual bacterial competition assays using the rat tissue cage model of intraperitoneal infection was performed exactly as described previously (48). In brief, overnight cultures in BHIS were diluted in PBS and mixed in a 1:1 ratio of parent strain/pNBU2-*ermG* (BER-208) to Δ*tonB3*/pNBU2-*bla-tetQ* (BER-209) at a total of approximately 1×10^5 CFU/mL for each strain as a standard inoculum. An aliquot of the inoculum suspension was serially diluted in PBS and plated on erythromycin (10 μg/mL) or tetracycline (5 μg/mL) to determine inoculum CFU/mL counts. Four milliliters of inoculum were injected into the intraperitoneal tissue cage of 3 rats per group in two independent experiments ($n = 6$). Fluid sample aspirates were taken at 1, 4, 8, and 15 days postinfection, serially diluted, and plated on media containing rifampin (20 μg/mL), gentamicin (100 μg/mL), FUdR (200 μg/mL), plus erythromycin (10 μg/mL) or tetracycline (5 μg/mL). After 3 to 4 days of incubation in an anaerobic chamber at 37°C, colonies from each sample time point were counted and normalized to CFU/mL of tissue cage serous fluid. The limit of detection was 1×10^1 CFU/mL.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.1 MB.

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REFERENCES

1. Schauer K, Rodionov DA, de Reuse H. 2008. New substrates for TonB-dependent transport: do we only see the "tip of the iceberg"? Trends Biochem Sci 33:330–338. https://doi.org/10.1016/j.tibs.2008.04.012.
2. Noinaj N, Guillier M, Barnard TJ, Buchanan SK. 2010. TonB-dependent transporters: regulation, structure, and function. Annu Rev Microbiol 64:43–60. https://doi.org/10.1146/annurev.micro.112408.134247.
3. Klebba PE, Newton SMC, Six DA, Kumar A, Yang T, Nairn BL, Munger C, Chakravorty S. 2021. Iron acquisition systems of Gram-negative bacterial pathogens define TonB-dependent pathways to novel antibiotics. Chem Rev 121:5193–5239. https://doi.org/10.1021/acs.chemrev.0c01005.
4. Braun V, Endriss F. 2007. Energy-coupled outer membrane transport proteins and regulatory proteins. Biometals 20:219–231. https://doi.org/10.1007/s10534-006-9072-5.
5. Krewulak KD, Vogel HJ. 2011. TonB or not TonB: is that the question? Biochem Cell Biol 89:87–97. https://doi.org/10.1139/o10-141.
6. Postle K, Larsen RA. 2007. TonB-dependent energy transduction between outer and cytoplasmic membranes. Biometals 20:453–465. https://doi.org/10.1007/s10534-006-9071-6.
7. Gresock MG, Kastead KA, Postle K. 2015. From homodimer to heterodimer and back: elucidating the TonB energy transduction cycle. J Bacteriol 197:3433–3445. https://doi.org/10.1128/JB.00484-15.
8. Maki-Yonekura S, Matsuoka R, Yamashita Y, Shimizu H, Tanaka M, Iwabuki F, Yonekura K. 2018. Hexameric and pentameric complexes of the ExbBD energizer in the Ton system. Elife 7:e35419. https://doi.org/10.7554/eLife.37833.
9. Lau CK, Krewulak KD, Vogel HJ. 2016. Bacterial ferrous iron transport: the Feo system. FEMS Microbiol Rev 40:273–298. https://doi.org/10.1093/femsre/fuv049.
10. Young GM, Postle K. 1994. Repression of *tonB* transcription during anaerobic growth requires Fur binding at the promoter and a second factor binding upstream. Mol Microbiol 11:943–954. https://doi.org/10.1111/j.1365-2958.1994.tb00373.x.
11. Lodge JS, Emery T. 1984. Anaerobic iron uptake by *Escherichia coli*. J Bacteriol 160:801–804. https://doi.org/10.1128/jb.160.2.801-804.1984.
12. Kwon O, Hudspeth ME, Meganathan R. 1996. Anaerobic biosynthesis of enterobactin *Escherichia coli*: regulation of *entC* gene expression and evidence against its involvement in menaquinone (vitamin K2) biosynthesis. J Bacteriol 178:3252–3259. https://doi.org/10.1128/jb.178.11.3252-3259.1996.
13. Troxell B, Fink RC, Porwollik S, McClelland M, Hassan HM. 2011. The Fur regulon in anaerobically grown *Salmonella enterica* sv. Typhimurium: identification of new Fur targets. BMC Microbiol 11:236. https://doi.org/10.1186/1471-2180-11-236.
14. Espey MG. 2013. Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. Free Radic Biol Med 55:130–140. https://doi.org/10.1016/j.freeradbiomed.2012.10.554.

15. Rivera-Chávez F, Lopez CA, Bäumler AJ. 2017. Oxygen as a driver of gut dysbiosis. Free Radic Biol Med 105:93–101. https://doi.org/10.1016/j.freeradbiomed.2016.09.022.
16. Friedman ES, Bittinger K, Esipova TV, Hou L, Chau L, Jiang J, Mesaros C, Lund PJ, Liang X, FitzGerald GA, Goulian M, Lee D, Garcia BA, Blair IA, Vinogradov SA, Wu GD. 2018. Microbes vs. chemistry in the origin of the anaerobic gut lumen. Proc Natl Acad Sci U S A 115:4170–4175. https://doi.org/10.1073/pnas.1718635115.
17. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, Hooper LV, Gordon JI. 2003. A genomic view of the human–*Bacteroides thetaiotaomicron* symbiosis. Science 299:2074–2076. https://doi.org/10.1126/science.1080029.
18. Koebnik R. 2005. TonB-dependent trans-envelope signalling: the exception or the rule? Trends Microbiol 13:343–347. https://doi.org/10.1016/j.tim.2005.06.005.
19. Cerdeño-Tárraga AM, Patrick S, Crossman LC, Blakely G, Abratt V, Lennard N, Poxton I, Duerden B, Harris B, Quail MA, Barron A, Clark L, Corton C, Doggett J, Holden MT, Larke N, Line A, Lord A, Norbertczak H, Ormond D, Price C, Rabbinowitsch E, Woodward J, Barrell B, Parkhill J. 2005. Extensive DNA inversions in the *B. fragilis* genome control variable gene expression. Science 307:1463–1465. https://doi.org/10.1126/science.1107008.
20. Patrick S, Blakely GW, Houston S, Moore J, Abratt VR, Bertalan M, Cerdeño-Tárraga AM, Quail MA, Corton N, Corton C, Bignell A, Barron A, Clark L, Bentley SD, Parkhill J. 2010. Twenty-eight divergent polysaccharide loci specifying within- and amongst-strain capsule diversity in three strains of *Bacteroides fragilis*. Microbiology (Reading) 156:3255–3269. https://doi.org/10.1099/mic.0.042978-0.
21. Bolam DN, van den Berg B. 2018. TonB-dependent transport by the gut microbiota: novel aspects of an old problem. Curr Opin Struct Biol 51:35–43. https://doi.org/10.1016/j.sbi.2018.03.001.
22. Pollet RM, Martin LM, Koropatkin NM. 2021. TonB-dependent transporters in the Bacteroidetes: Unique domain structures and potential functions. Mol Microbiol 115:490–501. https://doi.org/10.1111/mmi.14683.
23. Huang B, Ru K, Yuan Z, Whitchurch CB, Mattick JS. 2004. *tonB3* is required for normal twitching motility and extracellular assembly of type IV pili. J Bacteriol 186:4387–4399. https://doi.org/10.1128/JB.186.13.4387-4389.2004.
24. Chu BC, Peacock RS, Vogel HJ. 2007. Bioinformatic analysis of the TonB protein family. Biometals 20:467–483. https://doi.org/10.1007/s10534-006-9049-4.
25. Gómez-Santos N, Glatter T, Koebnik R, Świątek-Połatyńska MA, Søgaard-Andersen L. 2019. A TonB-dependent transporter is required for secretion of protease PopC across the bacterial outer membrane. Nat Commun 10:1360. https://doi.org/10.1038/s41467-019-09366-9.
26. Chatterjee A, O'Brian MR. 2018. Rapid evolution of a bacterial iron acquisition system. Mol Microbiol 108:90–100. https://doi.org/10.1111/mmi.13918.
27. Kuehl CJ, Crosa JH. 2010. The TonB energy transduction systems in *Vibrio* species. Future Microbiol 5:1403–1412. https://doi.org/10.2217/fmb.10.90.
28. Mey AR, Payne SM. 2001. Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. Mol Microbiol 42:835–849. https://doi.org/10.1046/j.1365-2958.2001.02683.x.
29. Dong Y, Geng J, Liu J, Pang M, Awan F, Lu C, Liu Y. 2019. Roles of three TonB systems in the iron utilization and virulence of the *Aeromonas hydrophila* Chinese epidemic strain NJ-35. Appl Microbiol Biotechnol 103:4203–4215. https://doi.org/10.1007/s00253-019-09757-4.
30. Fujita M, Mori K, Hara H, Hishiyama S, Kamimura N, Masai E. 2019. A TonB-dependent receptor constitutes the outer membrane transport system for a lignin-derived aromatic compound. Commun Biol 2:432. https://doi.org/10.1038/s42003-019-0676-z.
31. Zimbler DL, Arivett BA, Beckett AC, Menke SM, Actis LA. 2013. Functional features of TonB energy transduction systems of *Acinetobacter baumannii*. Infect Immun 81:3382–3394. https://doi.org/10.1128/IAI.00540-13.
32. Tsolis RM, Bäumler AJ, Heffron F, Stojiljkovic I. 1996. Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. Infect Immun 64:4549–4556. https://doi.org/10.1128/iai.64.11.4549-4556.1996.
33. Torres AG, Redford P, Welch RA, Payne SM. 2001. TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. Infect Immun 69:6179–6185. https://doi.org/10.1128/IAI.69.10.6179-6185.2001.
34. Bosch M, Garrido E, Llagostera M, Pérez de Rozas AM, Badiola I, Barbé J. 2002. *Pasteurella multocida exbB*, *exbD* and *tonB* genes are physically linked but independently transcribed. FEMS Microbiol Lett 210:201–208. https://doi.org/10.1111/j.1574-6968.2002.tb11181.x.
35. Costa LF, Mol JP, Silva AP, Macêdo AA, Silva TM, Alves GE, Winter S, Winter MG, Velazquez EM, Byndloss MX, Bäumler AJ, Tsolis RM, Paixão TA, Santos RL. 2016. Iron acquisition pathways and colonization of the inflamed intestine by *Salmonella enterica* serovar Typhimurium. Int J Med Microbiol 306:604–610. https://doi.org/10.1016/j.ijmm.2016.10.004.
36. Abdelhamed H, Lawrence ML, Karsi A. 2017. The role of TonB gene in *Edwardsiella ictaluri* virulence. Front Physiol 8:1066. https://doi.org/10.3389/fphys.2017.01066.
37. Runci F, Gentile V, Frangipani E, Rampioni G, Leoni L, Lucidi M, Visaggio D, Harris G, Chen W, Stahl J, Averhoff B, Visca P. 2019. Contribution of active iron uptake to *Acinetobacter baumannii* pathogenicity. Infect Immun 87:e00755-18. https://doi.org/10.1128/IAI.00755-18.
38. Martens EC, Lowe EC, Chiang H, Pudlo NA, Wu M, McNulty NP, Abbott DW, Henrissat B, Gilbert HJ, Bolam DN, Gordon JI. 2011. Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. PLoS Biol 9:e1001221. https://doi.org/10.1371/journal.pbio.1001221.
39. Martens EC, Kelly AG, Tauzin AS, Brumer H. 2014. The devil lies in the details: how variations in polysaccharide fine-structure impact the physiology and evolution of gut microbes. J Mol Biol 426:3851–3865. https://doi.org/10.1016/j.jmb.2014.06.022.
40. Cockburn DW, Koropatkin NM. 2016. Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease. J Mol Biol 428:3230–3252. https://doi.org/10.1016/j.jmb.2016.06.021.
41. Rocha ER, Bergonia HA, Gerdes S, Jeffrey Smith C. 2019. *Bacteroides fragilis* requires the ferrous-iron transporter FeoAB and the CobN-like proteins BtuS1 and BtuS2 for assimilation of iron released from heme. Microbiologyopen 8:e00669. https://doi.org/10.1002/mbo3.669.
42. Brown HA, Koropatkin NM. 2021. Host glycan utilization within the Bacteroidetes Sus-like paradigm. Glycobiology 31:697–706. https://doi.org/10.1093/glycob/cwaa054.
43. Glowacki RWP, Martens EC. 2020. If you eat it, or secrete it, they will grow: the expanding list of nutrients utilized by human gut bacteria. J Bacteriol 203:e00481-20. https://doi.org/10.1128/JB.00481-20.
44. Cao Y, Rocha ER, Smith CJ. 2014. Efficient utilization of complex N-linked glycans is a selective advantage for *Bacteroides fragilis* in extraintestinal infections. Proc Natl Acad Sci U S A 111:12901–12906. https://doi.org/10.1073/pnas.1407344111.
45. Spence C, Wells WG, Smith CJ. 2006. Characterization of the primary starch utilization operon in the obligate anaerobe *Bacteroides fragilis*: regulation by carbon source and oxygen. J Bacteriol 188:4663–4672. https://doi.org/10.1128/JB.00125-06.
46. Rocha ER, Krykunivsky AS. 2017. Anaerobic utilization of Fe(III)-xenosiderophores among *Bacteroides* species and the distinct assimilation of Fe(III)-ferrichrome by *Bacteroides fragilis* within the genus. MicrobiologyOpen 6:e479. https://doi.org/10.1002/mbo3.479.
47. Otto BR, Sparrius M, Verweij-van Vught AM, MacLaren DM. 1990. Iron-regulated outer membrane protein of *Bacteroides fragilis* involved in heme uptake. Infect Immun 58:3954–3958. https://doi.org/10.1128/iai.58.12.3954-3958.1990.
48. Parker AC, Bergonia HA, Seals NL, Baccanale CL, Rocha ER. 2020. The *uroS* and *yifB* genes conserved among tetrapyrrole synthesizing-deficient *Bacteroidales* are involved in *Bacteroides fragilis* heme assimilation and survival in experimental intraabdominal infection and intestinal colonization. Infect Immun 88:e00103-20. https://doi.org/10.1128/IAI.00103-20.
49. Madej M, White JBR, Nowakowska Z, Rawson S, Scavenius C, Enghild JJ, Bereta GP, Pothula K, Kleinekathoefer U, Baslé A, Ranson NA, Potempa J, van den Berg B. 2020. Structural and functional insights into oligopeptide acquisition by the RagAB transporter from *Porphyromonas gingivalis*. Nat Microbiol 5:1016–1025. https://doi.org/10.1038/s41564-020-0716-y.
50. Glenwright AJ, Pothula KR, Bhamidimarri SP, Chorev DS, Baslé A, Firbank SJ, Zheng H, Robinson CV, Winterhalter M, Kleinekathöfer U, Bolam DN, van den Berg B. 2017. Structural basis for nutrient acquisition by dominant members of the human gut microbiota. Nature 541:407–411. https://doi.org/10.1038/nature20828.
51. Mey AR, Payne SM. 2003. Analysis of residues determining specificity of *Vibrio cholerae* TonB1 for its receptors. J Bacteriol 185:1195–1207. https://doi.org/10.1128/JB.185.4.1195-1207.2003.
52. Gresock MG, Postle K. 2017. Going outside the TonB Box: identification of novel FepA-TonB interactions *in vivo*. J Bacteriol 199:e00649-16. https://doi.org/10.1128/JB.00649-16.
53. Devanathan S, Postle K. 2007. Studies on colicin B translocation: FepA is gated by TonB. Mol Microbiol 65:441–453. https://doi.org/10.1111/j.1365-2958.2007.05808.x.
54. Celia H, Noinaj N, Zakharov SD, Bordignon E, Botos I, Santamaria M, Barnard TJ, Cramer WA, Lloubes R, Buchanan SK. 2016. Structural insight into the role

of the Ton complex in energy transduction. Nature 538:60–65. https://doi.org/10.1038/nature19757.
55. Kashket ER. 1981. Proton motive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. J Bacteriol 146:369–776. https://doi.org/10.1128/jb.146.1.369-376.1981.
56. Kashket ER. 1981. Effects of aerobiosis and nitrogen source on the proton motive force in growing *Escherichia coli* and *Klebsiella pneumoniae* cells. J Bacteriol 146:377–384. https://doi.org/10.1128/jb.146.1.377-384.1981.
57. Goodwin S, Zeikus JG. 1987. Physiological adaptations of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi*. J Bacteriol 169:2150–2157. https://doi.org/10.1128/jb.169.5.2150-2157.1987.
58. Tran QH, Unden G. 1998. Changes in the proton potential and the cellular energetics of *Escherichia coli* during growth by aerobic and anaerobic respiration or by fermentation. Eur J Biochem 251:538–543. https://doi.org/10.1046/j.1432-1327.1998.2510538.x.
59. Russell JB, Diez-Gonzalez F. 1998. The effects of fermentation acids on bacterial growth. Adv Microb Physiol 39:205–234. https://doi.org/10.1016/s0065-2911(08)60017-x.
60. Trchounian A, Trchounian K. 2019. Fermentation revisited: How do microorganisms survive under energy-limited conditions? Trends Biochem Sci 44:391–400. https://doi.org/10.1016/j.tibs.2018.12.009.
61. Larsen RA, Thomas MG, Postle K. 1999. Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol Microbiol 31:1809–1824. https://doi.org/10.1046/j.1365-2958.1999.01317.x.
62. Kaserer WA, Jiang X, Xiao Q, Scott DC, Bauler M, Copeland D, Newton SM, Klebba PE. 2008. Insight from TonB hybrid proteins into the mechanism of iron transport through the outer membrane. J Bacteriol 190:4001–4016. https://doi.org/10.1128/JB.00135-08.
63. Jordan LD, Zhou Y, Smallwood CR, Lill Y, Ritchie K, Yip WT, Newton SM, Klebba PE. 2013. Energy-dependent motion of TonB in the Gram-negative bacterial inner membrane. Proc Natl Acad Sci U S A 110:11553–11558. https://doi.org/10.1073/pnas.1304243110.
64. Rollauer SE, Sooreshjani MA, Noinaj N, Buchanan SK. 2015. Outer membrane protein biogenesis in Gram-negative bacteria. Philos Trans R Soc B 370:20150023. https://doi.org/10.1098/rstb.2015.0023.
65. Konovalova A, Kahne DE, Silhavy TJ. 2017. Outer membrane biogenesis. Annu Rev Microbiol 71:539–556. https://doi.org/10.1146/annurev-micro-090816-093754.
66. Diederichs KA, Buchanan SK, Botos I. 2021. Building better barrels—β-barrel biogenesis and insertion in bacteria and mitochondria. J Mol Biol 433:166894. https://doi.org/10.1016/j.jmb.2021.166894.
67. Kulp A, Kuehn MJ. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol 64:163–184. https://doi.org/10.1146/annurev.micro.091208.073413.
68. Haurat MF, Elhenawy W, Feldman MF. 2015. Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. Biol Chem 396:95–109. https://doi.org/10.1515/hsz-2014-0183.
69. YashRoy RC. 2017. Outer membrane vesicles of Gram-negative bacteria: nanoware for combat against microbes and macrobes, p. 341–367. *In* Ficai A, Grumezescu AM (ed), Micro and nano technologies, nanostructures for antimicrobial therapy. Elsevier, Amsterdam, The Netherlands. https://doi.org/10.1016/B978-0-323-46152-8.00015-9.
70. Higgs PI, Letain TE, Merriam KK, Burke NS, Park H, Kang C, Postle K. 2002. TonB interacts with nonreceptor proteins in the outer membrane of *Escherichia coli*. J Bacteriol 184:1640–1648. https://doi.org/10.1128/JB.184.6.1640-1648.2002.
71. Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harb Perspect Biol 2:a000414. https://doi.org/10.1101/cshperspect.a000414.
72. Doyle MT, Bernstein HD. 2021. BamA forms a translocation channel for polypeptide export across the bacterial outer membrane. Mol Cell 81:2000–2012.e3. https://doi.org/10.1016/j.molcel.2021.02.023.
73. Patrick S, McKenna JP, O'Hagan S, Dermott E. 1996. A comparison of the haemagglutinating and enzymic activities of *Bacteroides fragilis* whole cells and outer membrane vesicles. Microb Pathog 20:191–202. https://doi.org/10.1006/mpat.1996.0018.
74. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. 2012. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell Host Microbe 12:509–520. https://doi.org/10.1016/j.chom.2012.08.004.
75. Jones EJ, Booth C, Fonseca S, Parker A, Cross K, Miquel-Clopés A, Hautefort I, Mayer U, Wileman T, Stentz R, Carding SR. 2020. The uptake, trafficking, and biodistribution of *Bacteroides thetaiotaomicron* generated outer membrane vesicles. Front Microbiol 11:57. https://doi.org/10.3389/fmicb.2020.00057.
76. Dutton RJ, Boyd D, Berkmen M, Beckwith J. 2008. Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. Proc Natl Acad Sci U S A 105:11933–11938. https://doi.org/10.1073/pnas.0804621105.
77. Rocha ER, Tzianabos AO, Smith CJ. 2007. Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe *Bacteroides fragilis*. J Bacteriol 189:8015–8023. https://doi.org/10.1128/JB.00714-07.
78. Reott MA, Parker AC, Rocha ER, Smith CJ. 2009. Thioredoxins in redox maintenance and survival during oxidative stress of *Bacteroides fragilis*. J Bacteriol 191:3384–3391. https://doi.org/10.1128/JB.01665-08.
79. Shouldice SR, Cho SH, Boyd D, Heras B, Eser M, Beckwith J, Riggs P, Martin JL, Berkmen M. 2010. *In vivo* oxidative protein folding can be facilitated by oxidation-reduction cycling. Mol Microbiol 75:13–28. https://doi.org/10.1111/j.1365-2958.2009.06952.x.
80. Tang YP, Dallas MM, Malamy MH. 1999. Characterization of the *BatI* (Bacteroides aerotolerance) operon in *Bacteroides fragilis*: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in in vivo model systems. Mol Microbiol 32:139–149. https://doi.org/10.1046/j.1365-2958.1999.01337.x.
81. Martens EC, Chiang HC, Gordon JI. 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe 4:447–457. https://doi.org/10.1016/j.chom.2008.09.007.
82. Pudlo NA, Urs K, Kumar SS, German JB, Mills DA, Martens EC. 2015. Symbiotic human gut bacteria with variable metabolic priorities for host mucosal glycans. mBio 6:e01282-15–e01215. https://doi.org/10.1128/mBio.01282-15.
83. Otto BR, van Dooren SJ, Dozois CM, Luirink J, Oudega B. 2002. *Escherichia coli* hemoglobin protease autotransporter contributes to synergistic abscess formation and heme-dependent growth of *Bacteroides fragilis*. Infect Immun 70:5–10. https://doi.org/10.1128/IAI.70.1.5-10.2002.
84. Murima P, McKinney JD, Pethe K. 2014. Targeting bacterial central metabolism for drug development. Chem Biol 21:1423–1432. https://doi.org/10.1016/j.chembiol.2014.08.020.
85. Feng X, Zhu W, Schurig-Briccio LA, Lindert S, Shoen C, Hitchings R, Li J, Wang Y, Baig N, Zhou T, Kim BK, Crick DC, Cynamon M, McCammon JA, Gennis RB, Oldfield E. 2015. Antiinfectives targeting enzymes and the proton motive force. Proc Natl Acad Sci U S A 112:E7073–E7082. https://doi.org/10.1073/pnas.1521988112.
86. Nairn BL, Eliasson OS, Hyder DR, Long NJ, Majumdar A, Chakravorty S, McDonald P, Roy A, Newton SM, Klebba PE. 2017. Fluorescence high-throughput screening for inhibitors of TonB action. J Bacteriol 199:e00889-16. https://doi.org/10.1128/JB.00889-16.
87. Rocha ER, Smith CJ. 2004. Transcriptional regulation of the *Bacteroides fragilis* ferritin gene (*ftnA*) by redox stress. Microbiology (Reading) 150:2125–2134. https://doi.org/10.1099/mic.0.26948-0.
88. Varel VH, Bryant MP. 1974. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. Appl Microbiol 28:251–2577. https://doi.org/10.1128/am.28.2.251-257.1974.
89. Rocha ER, de Uzeda M, Brock JH. 1991. Effect of ferric and ferrous iron chelators on growth of *Bacteroides fragilis* under anaerobic conditions. FEMS Microbiol Lett 68:45–50. https://doi.org/10.1016/0378-1097(91)90393-o.
90. Sperry JF, Appleman MD, Wilkins TD. 1977. Requirement of heme for growth of *Bacteroides fragilis*. Appl Environ Microbiol 34:386–390. https://doi.org/10.1128/aem.34.4.386-390.1977.
91. National Research Council. 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
92. Simon R, Priefer U, Pühler A. 1983. A broad range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat Biotechnol 1:784–791. https://doi.org/10.1038/nbt1183-784.
93. Koropatkin NM, Martens EC, Gordon JI, Smith TJ. 2008. Starch catabolism by a prominent human gut symbiont is directed by the recognition of amylose helices. Structure 16:1105–1015. https://doi.org/10.1016/j.str.2008.03.017.