The *uroS* and *yifB* Genes Conserved among Tetrapyrrole Synthesizing-Deficient *Bacteroidales* Are Involved in *Bacteroides fragilis* Heme Assimilation and Survival in Experimental Intra-abdominal Infection and Intestinal Colonization

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ABSTRACT The human intestinal anaerobic commensal and opportunistic pathogen *Bacteroides fragilis* does not synthesize the tetrapyrrole protoporphyrin IX in order to form heme that is required for growth stimulation and survival *in vivo*. Consequently, *B. fragilis* acquires essential heme from host tissues during extraintestinal infection. The absence of several genes necessary for *de novo* heme biosynthesis is a common characteristic of many anaerobic bacteria; however, the *uroS* gene, encoding a uroporphyrinogen III synthase for an early step of heme biosynthesis, is conserved among the heme-requiring *Bacteroidales* that inhabit the mammalian gastrointestinal tract. In this study, we show that the ability of *B. fragilis* to utilize heme or protoporphyrin IX for growth was greatly reduced in a $\Delta uroS$ mutant. This growth defect appears to be linked to the suppression of reverse chelatase and ferrochelatase activities in the absence of *uroS*. In addition, this $\Delta uroS$ suppressive effect was enhanced by the deletion of the *yifB* gene, which encodes an Mg^{2+} -chelatase protein belonging to the ATPases associated with various cellular activities (AAA⁺) superfamily of proteins. Furthermore, the $\Delta uroS$ mutant and the $\Delta uroS \Delta yifB$ double mutant had a severe survival defect compared to the parent strain in competitive infection assays using animal models of intra-abdominal infection and intestinal colonization. This shows that the presence of the *uroS* and *yifB* genes in *B. fragilis* seems to be linked to pathophysiological and nutritional competitive fitness for survival in host tissues. Genetic complementation studies and enzyme kinetics assays indicate that *B. fragilis* UroS is functionally different from canonical bacterial UroS proteins. Taken together, these findings show that heme assimilation and metabolism in the anaerobe *B. fragilis* have diverged from those of aerobic and facultative anaerobic pathogenic bacteria.

KEYWORDS *Bacteroides fragilis*, intestinal colonization, UroS, YifB, anaerobic infection, ferrochelatase, heme assimilation, intra-abdominal infection, reverse chelatase, uroporphyrinogen III synthase

The *Bacteroides* species are major components of the human lower intestinal tract, constituting approximately 20% to 40% of the total bacteria, with numbers reaching 10^{11} to 10^{12} CFU/g of colonic content (1–3). Using the anaerobic, opportunistic, human pathogen *Bacteroides fragilis* as a model organism, several studies have shown that it has an essential requirement for both exogenous heme and inorganic iron for growth stimulation *in vitro* and *in vivo* (4–9). This group of host-associated anaerobic

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bacteria is heme auxotrophic, lacking several genes in the tetrapyrrole biosynthetic pathway to form protoporphyrin IX (PpIX) as the final precursor for the synthesis of heme in this organism (6). In addition to host-associated *Bacteroides*, other members of the human gut microbiota, such as *Firmicutes*, *Actinobacteria*, and *Euryarchaeota*, also lack complete tetrapyrrole and essential heme biosynthetic pathways (6, 10).

Despite the absence of several genes needed to synthesize tetrapyrrole precursors for heme biosynthesis, a few intermediary genes, such as the uroporphyrinogen III synthase gene *uroS* (formerly named *hemD*) and the oxygen-independent coproporphyrinogen dehydrogenase gene *cgdH* (formerly *hemN*), are conserved in *B. fragilis* and in many closely related anaerobic bacteria within the *Bacteroidales* order (6). In contrast, free-living aerobic species within the *Cytophagales* and *Flavobacteriales* orders contain the complete *de novo* heme biosynthetic pathway found in classical Gram-negative bacteria (6). UroS activity is a strategic step in tetrapyrrole macrocycle biosynthesis as it catalyzes the cyclization of the linear tetrapyrrole preuroporphyrinogen (1-hydroxymethylbilane) by inversion of the D ring to form the asymmetric cyclic tetrapyrrole uroporphyrinogen III, an essential isomer for the synthesis of hemes, chlorophylls, and cobalamins (11–13). However, the role of the *uroS* gene in the genomes of tetrapyrrole synthesizing-deficient *Bacteroidales* is not well understood since an exogenous source of heme or protoporphyrin IX is sufficient to support their growth.

Moreover, the lack of a tetrapyrrole macrocycle synthesis pathway is not a competitive disadvantage relative to the heme-prototrophic bacteria in the nutritionally complex and highly populated intestinal environment (6, 14). In fact, it is heme-synthesizing bacteria such as proteobacteria that are outnumbered by heme-requiring bacterial species such as *Bacteroides*, which have the ability to scavenge intestinal luminal heme from host tissues, dietary sources, and bacterial sources (3, 6, 14, 15). Therefore, the conserved presence of the *uroS* gene in *Bacteroides* species may contribute to the ability of these organisms to regulate and utilize heme to compete effectively with other bacteria in extraintestinal infections and in the intestinal tract.

In this study, we investigated the participation of UroS in the utilization of heme and protoporphyrin IX by *B. fragilis* *in vitro* and survival *in vivo* in a rat tissue cage model of intraperitoneal infection and in a mouse intestinal colonization model. Moreover, during the course of this investigation, we initiated studies to show that the magnesium chelatase-like AAA⁺ (ATPases associated with diverse cellular activities) ATPase YifB acts in combination with UroS for survival *in vivo*. AAA⁺ proteins form a large and diverse superfamily found in all organisms, and they are involved in a wide variety of different functions (16–18). The YifB family is widespread in bacteria, and sequence analyses show that YifB is most closely related to the chelatase family, with which it shares distinct sequence signatures (19). Therefore, our objective is to elucidate the role of the *uroS* and *yifB* genes in the pathophysiology and ecological competitiveness of *B. fragilis*, which will help to advance our understanding of the mechanism developed by *B. fragilis* to adapt and survive as the opportunistic pathogen most frequently isolated from human infections caused by anaerobic bacteria.

RESULTS

Incomplete heme biosynthesis pathway genes. The *uroS* (*hemD*) gene is present in nearly all host-associated *Bacteroidales* genomes available in the GenBank database. The presence of *uroD* (*hemE*), *cgdH* (*hemN*), and *pgoX* (*hemY*) is also found in several species although less frequently (Fig. 1). All the host-associated *Bacteroidales* genomes lack the first four genes required for the synthesis of the UroS precursor hydroxymethylbilane (HMB): *gtrR* (*hemA*), *gsaM* (*hemL*), *pbgS* (*hemB*), and *hmbS* (*hemC*). In contrast, aerobic free-living *Bacteroidetes* members from the *Flavobacteriales* and *Cytophagales* orders possess the complete heme biosynthetic pathway. The oxygen-dependent coproporphyrinogen III oxidase gene, *cgdC* (*hemF*), is found in major free-living, heme-prototrophic species such as *Cytophaga hutchinsonii* and *Flavobacterium johnsoniae* but is absent in the heme-requiring, host-associated, obligately anaerobic species of the *Bacteroidales* order. Except for *Porphyromonas gingivalis*, all other

FIG 1 Schematic diagram of the conserved heme biosynthesis pathway genes in representative species within the *Bacteroidetes* phylum. (A) The classical heme biosynthetic pathway in most Gram-negative bacteria was used as a model for comparison with the *Bacteroidetes*. The enzymes' new designations, as described by Dailey et al. (10), are depicted in bold letters above the old abbreviations shown within parentheses. (B) The order of the arrows depicting the gene ORF locus is shown according to their respective product name in the heme biosynthetic pathway for the purpose of comparative analysis and does not represent their chromosomal genetic organization. Each gene is represented by an arrow with different colors and shades. Arrows positioned in a vertical column are predicted to encode gene products with homology to each respective enzyme on top of the column. (Republished from *Heme and iron metabolism in Bacteroides* [6].) The drawings are not to scale. GtrR, glutamyl-tRNA reductase (EC 1.2.1.70); GsaM, glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8); PbgS, porphobilinogen synthase (EC 4.2.1.24); HmbS, hydroxymethylbilane synthase (EC 2.5.1.61); UroS, uroporphyrinogen III synthase (EC 4.2.1.75); UroD, uroporphyrinogen decarboxylase (EC 4.1.1.37); CgdC, oxygen-dependent coproporphyrinogen III oxidase (EC 1.3.3.3); CgdH, oxygen-independent coproporphyrinogen III dehydrogenase (EC 1.3.99.22); PgdH1, protoporphyrinogen oxidase (EC 1.3.3.4); PgoX, protoporphyrinogen oxidase (EC 1.3.3.4); PpfC, protoporphyrin ferrochelatase (EC 4.99.1.1). The alternative routes of heme biosynthesis via the intermediates percorrin-2, sirohydrochlorin, siroheme, 12,18-didecarboxysiroheme, and iron-coproporphyrin III (Fe-COPRO III) in *Archaea* and *Desulfovibrio* (48–51) and via coproporphyrinogen oxidase (CgoX) in Gram-positive bacteria (52) are not shown for clarity since they do not appear to be present in *Bacteroides* spp. Bacterial names and strain designations are shown for each comparative row. B., *Bacteroides*; P., *Parabacteroides*.

species of the host-associated *Bacteroidales* lack the classical ferrochelatase gene *ppfC* (*hemH*).

***Bacteroides* species UroS is phylogenetically distinct from those of other bacteria.** An unrooted phylogenetic tree (Fig. 2) was obtained from a multiple-sequence alignment of *Bacteroidetes* UroS amino acid sequences with UroS sequences from other prokaryotes, archaea, and eukaryotes (see Fig. S1 in the supplemental material). The clusters with the *Bacteroides* spp. are noticeably separated from other bacteria. Archaea, eukarya, and all other bacteria are distinctly branched from a monophyletic clade separated from the branch related to aerobic, free-living, and heme-prototrophic species belonging to the *Flavobacteriales* and *Cytophagales* orders within the *Bacteroidetes* phylum. A comparison of the amino acid sequence alignments revealed that 7 out of the 10 conserved residues in the proposed active-site cleft of human UroS (Thr62, Ser63, Y168, S197, Lys220, T227, and T228) (20) are conserved in most of the *Bacteroides* species UroS proteins (Fig. S1). The Arg65 residue (human UroS), defined as being nonconserved in the active

FIG 2 Phylogenetic relationship of 72 uroporphyrinogen III synthase (UroS/HemD) homologue representatives from the *Bacteroidetes* phylum, aerobic and facultative bacteria (belonging to *Firmicutes*, cyanobacteria, proteobacteria, and deinococcus groups), *Archaea*, and *Eukaryota*. The unrooted phylogenetic tree was constructed from a multiple-amino-acid-sequence alignment based on the ClustalW algorithm in the AlignX program of Vector NTI 11.5.5. The neighbor-joining method of Saitou and Nei (53) was used to calculate the distances between all pairs, and the calculated relative distance values are depicted in parentheses. GenBank accession numbers for each protein entry are shown in square brackets following each organism's species designation.

site (20), is replaced mostly by His or Thr in the *Bacteroidetes*. In addition, the preceding residue, Pro64 (human UroS), is replaced by Arg in nearly all the examined *Bacteroidetes* UroS proteins (Fig. S1). Furthermore, the key conserved tyrosine residue Tyr168 (human UroS) or Tyr155 (*Thermus thermophilus* UroS), which may function in the removal of the C₂₀ hydroxyl of the substrate hydroxymethylbilane (13, 20), is conserved in all the *Bacteroidetes* phylum UroS proteins analyzed except for a Phe164 substitution in *Parabacteroides distasonis* ATCC 8503.

Phylogenetic analysis of *Bacteroides* species YifB. An unrooted phylogenetic tree (Fig. S2) constructed from multiple-sequence alignments of *Bacteroides* species YifB amino acid sequences with YifB sequences from other prokaryotes (Fig. S3) revealed that the clusters containing YifB from alpha-, beta-, gamma-, delta-, and epsilonproteobacteria; cyanobacteria; *Chlorobi* bacteria; cyanobacteria; actinobacteria; and *Clostridium* spp. are all branched from a distinct monophyletic clade divided between aerobic free-living groups within the *Bacteroidetes* phylum such as *Cytophaga* and *Flavobacterium*.

Role of UroS and YifB in growth *in vitro*. To test whether the lack of the *uroS* gene would affect *B. fragilis* physiology, the growths of the Δ uroS mutant (BER-184), the Δ yifB mutant (BER-185), and the Δ uroS Δ yifB double mutant (BER-186) strains were compared to that of the parent strain (Fig. 3A and B). When bacterial strains were grown in the presence of PpIX and inorganic ferrous iron or in the presence of heme as the sole source of iron, the Δ uroS and Δ uroS Δ yifB strains showed a strong growth defect compared to the parent strain. In contrast, the deletion of the *yifB* gene did not appear

FIG 3 Growth of *B. fragilis* strains in defined medium (DM) containing 0.5% glucose (A and B) or 0.3% starch (C and D). Media were supplemented with 5 μ g/ml protoporphyrin IX (PplX) plus 100 μ M ammonium ferrous sulfate (Fe) (A and C) or 5 μ g/ml hemin (He) plus 400 μ M bathophenanthroline disulfonic acid (BPS) (B and D). Strain designations are depicted as follows: parent (BER-183), $\Delta uroS$ (BER-184), $\Delta yifB$ (BER-185), $\Delta uroS \Delta yifB$ (BER-186), parent/pFD1045 (BER-201), $\Delta uroS$ /pFD1274 (BER-202), and $\Delta uroS \Delta yifB$ /pFD1274 (BER-203). Growth on starch in panels C and D was performed to induce the expression of the *uroS* gene in the pFD1274 plasmid derived from pFD1045. Vertical bars denote standard deviations of the means from two independent experiments in duplicate.

to affect growth compared to the parent strain under the same conditions (Fig. 3A and B). The genetic complementation of the $\Delta uroS$ mutant (BER-202) and $\Delta uroS \Delta yifB$ double mutant (BER-203) strains with the *uroS* gene expressed under the control of the starch/maltose-inducible promoter of the *osu* operon in the pFD1274 plasmid restored growth to levels similar to the parent strain level in medium containing starch (Fig. 3C and D). Western blot analyses using rabbit polyclonal antibodies raised against the purified *B. fragilis* UroS (Bf-UroS)-6 $\times$ His recombinant protein confirmed the expression of the UroS protein in the genetically complemented strain BER-202 (Fig. S4).

Heterologous complementation of the *B. fragilis uroS* deletion mutant with the *E. coli uroS* gene does not restore the growth phenotype. When the *Escherichia coli uroS* (Ec-*uroS*) gene was expressed in the *B. fragilis* $\Delta uroS$ deletion mutant carrying pER-323 (BER-234), it did not restore growth compared to the *B. fragilis* $\Delta uroS$ strain expressing the native Bf-*uroS* gene in pER-320 (BER-236) (Fig. 4A). In fact, the presence of the *E. coli uroS* gene caused an increase in the growth defect of the *B. fragilis* $\Delta uroS$ strain in defined medium (DM) containing heme as a sole source of iron. The growth of the *B. fragilis* strains in supplemented brain heart infusion broth (BHIS medium) was not significantly affected after 10 h and 24 h of incubation, except for the suppression of BER-234 strain growth at mid-log phase (Fig. 4B). The expression of the *B. fragilis uroS* gene in the SASZ31Rif strain carrying pER-320 (EC334) or pER-337 (EC339) did not stimulate growth on plates containing Luria-Bertani (LB) medium (Fig. 4C). The SASZ31Rif strain carrying an empty vector (EC333) did not grow on LB plates after a 24-h incubation aerobically, as expected. In contrast, the SASZ31Rif strain complemented with the *E. coli uroS* gene in the plasmid pER-323 (EC335) or pER-325 (EC336) grew on LB plates incubated aerobically. BHI plates incubated anaerobically were used as the growth control for the SASZ31Rif derivative strains used in this study (Fig. 4D).

Ferrochelataase and reverse chelataase activities. When crude extracts of bacteria grown on defined medium containing PplX and iron were analyzed, there were statistically significant decreases in ferrochelataase activity of approximately 3.5-fold in

FIG 4 Heterologous genetic complementation of the *B. fragilis* *uroS* deletion mutant and *E. coli* *uroS* mutant strains shows that *B. fragilis* *UroS* and *E. coli* *UroS* are functionally different. (A) Growth of *B. fragilis* strains in defined medium (DM) containing 0.5% glucose supplemented with 5 μ g/ml hemin (He) plus 400 μ M bathophenanthroline disulfonic acid (BPS). (B) Growth of *B. fragilis* strains in BHIS medium. Strains and the respective plasmids are depicted on the panels as follows: parent/pFD340 empty vector (BER-237), $\Delta uroS$ /pFD340 empty vector (BER-238), $\Delta uroS$ /pER-320 carrying the *Bf-uroS* construct (BER-236), and $\Delta uroS$ /pER-323 carrying *Ec-uroS_{BFRBS}* (BER-234). (C and D) Growth of the *E. coli* SASZ31Rif strain carrying different plasmid constructs in Luria-Bertani (LB) medium incubated aerobically (C) or in BHI medium containing 0.5% yeast extract (YE) and 1% glucose incubated anaerobically (D). Plasmid designations are depicted on each panel as follows: pFD340 empty vector (EC333), pER-320 carrying *Bf-uroS* (EC334), pER-323 carrying *Ec-uroS_{BFRBS}* (EC335), pER-325 carrying *Ec-uroS* (EC336), and pER-337 carrying *Bf-uroS_{ECRBS}* (EC339). Vertical bars denote standard deviations of the means from three independent experiments in triplicate.

the $\Delta uroS$ mutant (493 pmol/mg/h) and 3.2-fold (544 pmol/mg/h) in the $\Delta uroS$ $\Delta yifB$ double mutant compared to the parent strain (1,719 nmol/mg/h) (Fig. 5A). This indicated that the lack of the *uroS* gene led to a reduction in ferrochelatase activity. The genetic complementation of the $\Delta uroS$ strain ($\Delta uroS$ /pFD1274) restored ferrochelatase activity to approximately 2,390 pmol/mg/h, confirming the contribution of *UroS* to this process. Conversely, the lack of the *yifB* gene alone showed a significant increase in ferrochelatase activity of approximately 2.2-fold (3,876 pmol/mg/h) compared to the parent strain (Fig. 5A). The complementation of the *yifB* gene in the $\Delta yifB$ /pFD1272 strain did not alter the ferrochelatase activity (4,097 pmol/mg/h) seen in the $\Delta yifB$ strain in the presence of PpIX. However, in the presence of heme, the $\Delta yifB$ /pFD1272 strain showed enhanced ferrochelatase activity, as described below.

When bacteria were grown in defined medium supplemented with hemin as a sole source of iron (Fig. 5A), there were significant decreases in ferrochelatase activity of approximately 4.7-fold (1,422 pmol/mg/h) in the $\Delta uroS$ mutant (BER-184) and 4.6-fold (1,439 pmol/mg/h) in the $\Delta uroS$ $\Delta yifB$ double mutant (BER-186) compared to the parent strain (6,672 pmol/mg/h). The genetic complementation of *uroS* in the $\Delta uroS$ /pFD1274 (BER-202) strain increased activity to values approximately 2-fold (15,500 pmol/mg/h) higher than that in the parent strain, demonstrating that the presence of the *uroS* gene indeed affects ferrochelatase activity. Although the $\Delta yifB$ single mutant strain did not show significant differences in specific activity (7,183 pmol/mg/h) compared to the

FIG 5 Ferrochelatase and reverse chelatase (dechelate) activity assays of crude extracts of *Bacteroides fragilis* strains. Bacteria were grown in defined medium containing 5 μ g/ml protoporphyrin IX (PplX) plus 100 μ M ammonium ferrous sulfate or 5 μ g/ml hemin (He) plus 400 μ M bathophenanthroline disulfonic acid (BPS). Media were inoculated with a 1:50 dilution of a culture grown overnight in BHIS medium. Bacteria were then grown anaerobically for 24 to 48 h. Details of the ferrochelatase and reverse chelatase activity assay settings are described in Materials and Methods. Data are means $\pm$ standard deviations from two biologically independent experiments in triplicate. The significance (*P* value) by an unpaired *t* test (parametric and two tailed) between two experimental groups is shown above the horizontal bars. n.s., not significant.

parent strain, the genetically complemented $\Delta yifB$ /pFD1272 strain (BER-204) showed an increase of approximately 2.9-fold (19,581 pmol/mg/h) compared to the parent strain.

Figure 5B shows that the reverse chelatase activities were not significantly altered in either the $\Delta uroS$ mutant (2,502 pmol/mg/h), the $\Delta yifB$ mutant (1,065 pmol/mg/h), or the

$\Delta uroS \Delta yifB$ double mutant (2, 320 pmol/mg/h) strain compared to the parent strain (1,421 pmol/mg/h) when bacteria were grown in iron-replete medium containing PplX. The genetic complementation in the $\Delta uroS$ /pFD1274 and $\Delta yifB$ /pFD1272 strains did not significantly alter the enzyme activities in the presence of PplX. When the $\Delta uroS$ mutant strain was complemented with the entire putative polycistronic operon, containing the BF638R_0059, BF638R_0060, and BF638R_0061 (*uroS*) genes (Fig. S5) in the $\Delta uroS$ /pFD1276 strain (BER-212)(BF638R_0059 and BF638R_0060 are diploids), there was a significant increase in reverse chelatase activity of approximately 4-fold (5,228 pmol/mg/h) compared to the parent strain (1,421 pmol/h/mg).

When bacteria were grown in defined medium with heme as a sole source of iron, the mean reverse chelatase specific activities in the $\Delta uroS$ mutant (0.11 pmol/mg/h) and $\Delta yifB$ mutant (0.14 pmol/mg/h) strains were reduced by approximately 13-fold and 10-fold, respectively, compared to the parent strain (1.44 pmol/mg/h). However, these results were not statistically significant (Fig. 5C). In addition, the activity in the $\Delta uroS \Delta yifB$ double mutant strain was reduced to an undetectable level (below the blank level) compared to the parent strain. The genetically complemented strains showed restored reverse chelatase activity. However, the recombinant UroS-6 $\times$ His (rUroS-6 $\times$ His) protein purified under aerobic conditions did not show detectable forward or reverse chelatase activities (Fig. S6). The uroporphyrinogen III synthase specific activity of the rUroS-6 $\times$ His protein is 0.869 ± 0.042 nmol/mg/h, and the K_m for the substrate HMB is 0.064 ± 0.013 μ M.

To demonstrate whether or not the *uroS* and *yifB* genes would have a significant contribution to pathophysiology and survival in a host environment *in vivo*, experimental intra-abdominal infection and intestinal colonization studies were carried out.

UroS and YifB contribute to survival *in vivo*. (i) Intra-abdominal infection. Equal numbers of parent and mutant cells were used to coinfect rat tissue cages, and samples were taken over a time course. The surviving populations of the parent strain and the $\Delta uroS$ mutant strain revealed that the $\Delta uroS$ mutant was quickly outcompeted at 1 day postinfection, with an ~ 2 -log-fold decrease compared to the parent strain; i.e., $\sim 98\%$ of the total population was the parent strain (Fig. 6A). The $\Delta uroS$ mutant population decreased by >3 -log-fold by 15 days postinfection compared to the parent strain. This indicates that the lack of the *uroS* gene is a disadvantage regarding competitive fitness in an extraintestinal environment. When the $\Delta uroS \Delta yifB$ double mutant was coinoculated with the parent strain, the $\Delta uroS \Delta yifB$ population decreased by 2 logs at 1 day postinfection. Next, the $\Delta uroS \Delta yifB$ double mutant population steadily decreased at day 4 (2.7 logs), day 8 (3.5 logs), and day 15 (4 logs) postinfection compared to the parent strain, indicating that the mutation of the *yifB* gene in the $\Delta uroS \Delta yifB$ double mutant slightly enhanced the competitive disadvantage in growth compared to the parent strain (Fig. 6B). The $\Delta yifB$ single mutant was not tested for dual inoculation in intraperitoneal infection. Figure 6C shows that the parent/pNBU2-*bla-tetQ* and parent/pNBU2-*bla-ermG* strain populations compete equally, demonstrating that the insertion of the pNBU2-*bla-ermG* or pNBU2-*bla-tetQ* vector with the respective antibiotic markers did not affect bacterial recovery counts. We assume that single-copy insertions of the pNBU2-derived constructs were inserted into the *att1* or *att2* integration site in tRNA^{Ser} without disrupting genetic functions based on the nonreplicating properties of the NBU2 genetic element described in *Bacteroides thetaiotaomicron* (21).

The surviving numbers of parent strain and mutant strain cells determined for each rat were also used to calculate competitive indices. These results are shown in Fig. S7, where a competitive index score of 1 indicates that the mutant and wild type compete equally. The mean competitive index scores for the $\Delta uroS$ mutant were 0.03, 0.006, 0.002, and 0.0003 and the mean competitive index scores for the $\Delta uroS \Delta yifB$ mutant were 0.01, 0.002, 0.0005, and 0.0003 at days 1, 4, 8, and 15 postinfection, respectively. The competitive indices indicated that the $\Delta uroS \Delta yifB$ double mutant was slightly less competitive than the $\Delta uroS$ single mutant at days 1, 4, and 8 postinfection, although at day 15, both mutants were similarly less competitive than the parent strain.

FIG 6 Competitive survival of *B. fragilis* strains inoculated into the intraperitoneal tissue cage. (A) Dual infection with parent *Erm^r* (BER-208) versus Δ *uroS* *Tet^r* (BER-210) strains. (B) Dual infection with parent *Erm^r* (BER-208) versus Δ *uroS* Δ *yifB* *Tet^r* (BER-217) strains. (C) Dual-infection control with parent *Erm^r* (BER-208) versus parent *Tet^r* (BER-223) 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-bacterium suspension was 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.

(ii) Intestinal colonization. Dual colonization of the mouse intestinal tract with the parent strain and the Δ *uroS* mutant strain showed a decrease of approximately 2.5 logs in the Δ *uroS* mutant population compared to the parent strain after 7 days of colonization (Fig. 7A). The Δ *uroS* Δ *yifB* double mutant displayed a robust growth defect in the cecum, with an $\sim$ 4-log decrease (Fig. 7B), while the Δ *yifB* single mutant did not show a significant growth defect during the same colonization period compared to the parent strain (Fig. 7C).

For the purpose of comparison, we performed dual colonization with the BER-180 strain (*B. fragilis* 638R fusidic acid resistant) and the BER-51 strain (*B. fragilis* 638R Δ *feoAB::tetQ*) in mice placed on the same rodent diet. This normal standard diet is iron replete, containing 360 ppm iron (Prolab IsoPro RMH 300, product codes 5P75 and 5P76; LabDiet). Under these conditions, there was no significant difference in the population of the Δ *feoAB* mutant strain compared to the parent strain (Fig. 7D).

DISCUSSION

In this study, we show that a deletion of the *B. fragilis* *uroS* gene causes a severe growth defect compared to the parent strain both *in vivo* and *in vitro*. This indicates

FIG 7 Competitive dual bacterial colonization of the C57BL/6J mouse intestinal tract by *B. fragilis* strains. (A) Parent Erm^r (BER-208) versus Δ uroS Tet^r (BER-210) strains. (B) Parent Erm^r (BER-208) versus Δ uroS Δ yifB Tet^r (BER-217) strains. (C) Parent Erm^r (BER-208) versus Δ yifB Tet^r (BER-226) strains. (D) 638R FA^r (fusidic acid-resistant) (BER-180) versus Δ feoAB Tet^r (BER-51) strains. Erm^r is the erythromycin resistance marker in strains carrying pNBU2-*bla-ermG*, and Tet^r is the tetracycline resistance marker in strains carrying pNBU2-*bla-tetQ* except for Δ feoAB::*tetQ* strain BER-51. Two and four 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 1.1×10^{10} to 2.3×10^{10} CFU/ml of each strain by oral gavage as described in Materials and Methods. After 7 days, the cecal content was serially diluted and plated on BHIS medium containing appropriate antibiotics for the selection and enumeration of colonies. Colony counts were normalized to CFU per gram of cecal content. Mice were placed on a 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 (P value) following an unpaired t test (parametric and two tailed) for the two groups is shown above the horizontal bars. n.s., not significant.

that the conservation of an early intermediary gene in the heme biosynthetic pathway is necessary to endure the ecological pressure facing heme-requiring bacteria. Although heme or its precursor protoporphyrin IX is sufficient to maintain optimal growth and can be obtained from host tissues or dietary sources (6), the role of UroS in *B. fragilis* heme metabolism remains undefined. It is possible that *B. fragilis* UroS may function in a noncanonical way. There is little evidence that *B. fragilis* UroS acts as a genuine, primary uroporphyrinogen III synthase. This assumption is supported by the fact that the absence of the *uroS* and *yifB* genes compromises survival during extraintestinal infection and intestinal colonization compared to the parent strain. This indicates that these mutants were unable to obtain heme from host sources. Therefore, it is unlikely that these phenotypic properties were in any way associated with the conversion of hydroxymethylbilane into uroporphyrinogen III. This is based on the findings that the canonical *E. coli* UroS protein does not restore the growth of the *B. fragilis* Δ uroS deletion mutant strain, nor does *B. fragilis* UroS restore the growth of the *E. coli* *uroS* gene-deficient strain. This indicates that *B. fragilis* UroS and *E. coli* UroS may be functionally different.

B. fragilis UroS has a quite distant phylogenetic relationship to other bacterial UroS proteins. This agrees with the findings that *Bacteroides* spp. generally share little homology to other Gram-negative species, with amino acid identities of <40% being the most common (22). In addition, the UroS proteins have some of the most highly divergent amino acid sequences of the heme biosynthetic enzymes (13), although certain domains are highly conserved in several organisms (23). Moreover, the *B. fragilis* purified rUroS-6 $\times$ His protein uroporphyrinogen III synthase specific activity found in this study (0.869 ± 0.042 nmol/mg/h) is several orders of magnitude (6×10^5 to 1.7×10^6) lower than the specific activity of UroS reported for aerobic and facultative anaerobic bacteria such as *Bacillus subtilis* (565 μ mol/mg/h) (24) and *E. coli* (1,500 μ mol/mg/h) (25). In addition, *B. fragilis* rUroS-6 $\times$ His has a K_m (0.064 ± 0.013 μ M) approximately 5-fold and 78-fold lower than the K_m values of UroS from *B. subtilis* (K_m of 330 ± 30 nM) (24) and *E. coli* (K_m of 5 μ M) (25), respectively. These findings indicate that Bf-UroS binds the substrate HMB very efficiently, but the enzyme activity is very low compared to those of UroS proteins from *B. subtilis* and *E. coli*. The low Bf-UroS enzymatic activity, which indicates slow turnover or breakdown of the enzyme-substrate complex, may explain the inability of *B. fragilis* UroS to complement the *E. coli* UroS deficiency.

Nonetheless, our findings show that *B. fragilis* UroS has biochemical and molecular properties associated with other proteins and cellular processes involved in the chelate and reverse chelate activities whose mechanisms have yet to be characterized. In this regard, the *uroS* operon (see Fig. S5 in the supplemental material) contains two putative coexpressed, upstream genes that seem to affect reverse chelate activity when expressed in a multicopy plasmid in the presence of PplX. The *ygdH* gene (BF638R_0059) encodes a protein with a predicted NAD(P)-binding Rossmann fold domain, and the BF638R_0060 gene encodes a conserved, putative, integral membrane protein of unknown function with six transmembrane domains. Homology of BF638R_0060 is widespread among the *Bacteroides-Prevotella* group of heme-auxotrophic, host-associated anaerobes, and it has no significant homology to genes of any of the other major intestinal colonizers such as the *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. This indicates that the conserved genetic organization of the genes associated with *uroS* in *B. fragilis* performs metabolic activities that are yet to be defined. In addition, we cannot exclude from consideration that UroS may be involved in the molecular alterations of free heme to maintain it at nontoxic levels. Taken together, these findings indicate that tetrapyrrole metabolism, heme metabolism, and their regulation in heme-requiring, commensal bacteria and opportunistic, pathogenic, anaerobic bacteria have yet to be elucidated.

This study has also shown that the deletion of the *yifB* gene intensifies the survival defect of the Δ *uroS* mutant strain in extraintestinal infection and intestinal colonization. Unfortunately, very little is known about the function of the YifB family of proteins present in several bacterial families. YifB is a member of a subfamily of proteins of unknown function within the AAA⁺ ATPase superfamily. This class of proteins represents a distinct class of P-loop nucleoside triphosphatases (NTPases) that are involved in diverse functions in which the energy extracted from ATP hydrolysis is used for molecular remodeling events (16–19). Bf-YifB shares distinct amino acid sequence homology with Mg²⁺ chelate subunit I (Bchl/ChII) (44% identity and 62% similarity) from *Rhodobacter sphaeroides* and with the ATP-dependent Lon protease (44% identity and 62% similarity) from *E. coli*, which belongs to the large AAA⁺ ATPase superfamily. We do not have evidence for dual enzyme functionality, but our findings indicate that Bf-YifB is at least involved in chelate/dechelate activities, possibly by carrying out ATP hydrolysis to provide energy for the iron chelation/dechelation of heme anaerobically. Therefore, we believe that Bf-YifB may be divergent from the Bchl/ChII subunit, which, when associated with BchD/ChID, binds and hydrolyzes the ATP necessary for the incorporation of an Mg²⁺ ion into protoporphyrin IX by the catalytic BchH/ChIH subunit of the Mg²⁺ chelate complex (26, 27). Further investigations will help us to understand how Bf-YifB may contribute to the chelate/dechelate activities *in vitro* as well as in host tissues. It is plausible to speculate that the Bf-YifB and Bf-UroS mechanisms of action were developed and adapted to function anaerobically under the very low redox potential required by these organisms to grow. This supposition is based on the statement that “except for a few metabolic processes in which dioxygen serves as an essential reactant, the whole of metabolic biosynthesis in bacteria is anaerobic in character. This is clearly evidenced in strictly anaerobic bacteria capable of growth in minimal media which testifies to this fact” (28).

At this point in the investigation, it is not possible to explain the ecological benefit that the lack of the genes for *de novo* tetrapyrrole and heme biosynthesis achieves for the nutritional and pathophysiological attributes of *B. fragilis*. *B. fragilis* lacks GtrR (HemA), GsaM (HemL), PgbS (HemB), and HmbS (HemC) homologues for the synthesis of hydroxymethylbilane as a substrate precursor for UroS to form uroporphyrinogen III. Therefore, the significance of the conservation of UroS as well as YifB remains largely unknown. Some progress has been made to show that the ferrochelatase and reverse chelatase activities are decreased in the absence of the *uroS* and *yifB* genes; conversely, these activities are increased in the absence of the CobN-like BtuS1 and BtuS2 proteins (9). Moreover, the colonization fitness defects of the Δ *uroS* mutant and the Δ *uroS* Δ *yifB* double mutant strains were significantly greater than those of the Δ *feoAB* mutant

strain, indicating that heme assimilation and metabolism are important factors for survival, even under iron-replete conditions. These findings point to the fact that *B. fragilis* has developed unique regulatory and metabolic mechanisms to utilize heme that are divergent from those of other aerobic and facultative anaerobic bacteria.

The exploration and elucidation of the molecular mechanisms and regulatory systems that control heme utilization in *B. fragilis* pathogenesis will allow us to understand how intestinal, opportunistic pathogens rapidly adapt and compete for heme in extraintestinal infections and intestinal colonization. Investigations into metabolic pathways in heme-auxotrophic bacteria may identify new targets as a basis for the development of novel therapeutic interventions for anaerobic bacterial abscess infections that are currently difficult to treat as the number of antibiotic-resistant *Bacteroides* strains grows (29–31).

MATERIALS AND METHODS

Strains, media, and growth conditions. *B. fragilis* strains and plasmids used in this study are listed in Table 1. Strains were routinely grown on BHIS medium (brain heart infusion broth supplemented with L-cysteine [1 g/liter], hemin [5 mg/liter], and NaHCO₃ [20 ml of a 10% solution per liter]) or as otherwise stated in the text. Rifampin (20 µg/ml), gentamicin (100 µg/ml), tetracycline (5 µg/ml), and erythromycin (10 µg/ml) were added to the media when required. A modified defined medium (DM) (32) containing KH₂PO₄ (1.15 g/liter), NH₄SO₄ (0.4 g/liter), NaCl (0.9 g/liter), L-methionine (75 mg/liter), MgCl₂·6H₂O (20 mg/liter), CaCl₂·2H₂O (6.6 mg/liter), MnCl₂·4H₂O (1 mg/liter), CoCl₂·6H₂O (1 mg/liter), resazurin (1 mg/liter), L-cysteine (1 g/liter), hemin (5 mg/liter), and glucose (5 g/liter) was used. The final pH was 6.9. Twenty milliliters of sterile 10% NaHCO₃ was added per liter of medium inside the anaerobic chamber. For some experiments, soluble starch (0.3 g%) was added to replace glucose as an inducer of the *osu* promoter in strains carrying plasmids derived from pFD1045 (Table 1). Heme was replaced by protoporphyrin IX (5 µg/ml) as the source of the tetrapyrrole macrocycle when required (4). For iron restriction in DM, the ferrous iron chelator bathophenanthroline disulfonic acid (BPS) was added at a 400 µM final concentration. Ammonium ferrous sulfate at 100 µM was added for ferrous iron-replete growth conditions. *Escherichia coli* strains were grown routinely on Luria-Bertani medium supplemented with ampicillin (100 µg/ml), streptomycin (50 µg/ml), kanamycin (50 µg/ml), or tetracycline (10 µg/ml) when appropriate. The *E. coli* *uroS*-deficient strain SASZ31 (33) was grown anaerobically on BHI medium supplemented with yeast extract (0.5%) and D-glucose (1%). The SASZ31 strain forms dwarf colonies on BHI plates (33).

Construction of the *B. fragilis* 638R *tdk* deletion mutant strain. An 819-bp DNA fragment containing the upstream region plus 39 nucleotides of the N-terminal open reading frame (ORF) of the thymidine kinase gene (*tdk*) (BF638R_0630) was amplified from the *B. fragilis* 638R chromosome by PCR using primers NF-*tdk* and NR-*tdk* (see Table S1 in the supplemental material). The amplified fragment was cloned into the EcoRI and BamHI sites of the suicide vector pFD516 (34). An 850-bp DNA fragment containing the downstream region including 33 nucleotides of the C-terminal ORF was amplified using primers CF-*tdk* and CR-*tdk* and cloned into the BamHI and Sall sites of pFD516 containing the 819-bp N-terminal fragment to construct a 1,670-bp DNA fragment with a 528-bp internal deletion of the *tdk* gene. The new construct, pFD1241, was mobilized from *E. coli* DH10B into *B. fragilis* 638R by triparental mating (35). The transconjugants were selected on BHIS plates containing rifampin (20 µg/ml), gentamicin (100 µg/ml), and erythromycin (10 µg/ml). Resolution of Δ *tdk* mutants was obtained by growing four transconjugants in BHIS broth containing rifampin (20 µg/ml) and 5-fluor-2'-deoxyuridine (FUDR) (200 µg/ml). The use of FUDR was performed according to the concentration used for positive counterselection of the *Bacteroides thetaiotaomicron* Δ *tdk* strain (36). The Δ *tdk* strain-enriched cultures were plated out on BHIS plates containing rifampin (20 µg/ml) and FUDR (200 µg/ml). Colonies were tested for erythromycin sensitivity to confirm the loss of the suicide vector. The FUDR-resistant and erythromycin-sensitive strains were selected for PCR analysis using primers NF-*tdk* and CR-*tdk* to confirm the deletion of a 528-bp internal DNA fragment of the *tdk* gene. The BER-183 strain (BF638R Δ *tdk*) was used as the parent strain for further studies.

Construction of the *uroS* deletion mutant. DNA fragments of 1,581 bp and 1,629 bp containing the upstream N-terminal and downstream C-terminal DNA fragment regions of the *uroS* gene (BF638R_0061) were amplified by PCR from the *B. fragilis* 638R chromosome using the primer sets UroS-NT-XbaI/UroS-NT-SEW and UroS-CT-SEW/UroS-CT-Sall, respectively. The N-terminal and C-terminal DNA fragments were joined together using a "sewing" PCR methodology. The 3,210-bp DNA fragment product containing the 429-bp internal deletion of the *uroS* gene was reamplified by PCR using primers UroS-NT-XbaI and UroS-CT-Sall. The amplified fragment was cloned into the XbaI and Sall sites of the positive counterselection suicide vector pExchange-*tdk* (36). The new construct, pER-302, was mobilized from *E. coli* S17-1 λ pir into BER-183 by biparental mating. Transconjugants were selected on BHIS plates containing rifampin (20 µg/ml), gentamicin (100 µg/ml), and erythromycin (10 µg/ml). Transconjugants were grown on BHIS broth with rifampin and gentamicin overnight, anaerobically at 37°C, to enrich for double-crossover genetic exchange recombinants. The cultures were plated on BHIS plates containing rifampin (20 µg/ml), gentamicin (100 µg/ml), and FUDR (200 µg/ml). Sensitivity to erythromycin was assessed to confirm the loss of the suicide vector. PCR amplification using primers UroS-mutcheck-F and UroS-mutcheck-R was performed to confirm the deletion of the *uroS* gene in the new strain BER-184.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype ^a	Reference or source
Strains		
<i>B. fragilis</i> 638R	Clinical isolate; Rif ^r	54
BER-51	638R Δ feoAB::tetQ; Rif ^r Tet ^r	7
BER-180	638R spontaneous fusidic acid-resistant isolate	This study
BER-183	638R Δ tdk; Rif ^r FUDR ^r	This study
BER-184	638R Δ tdk Δ uroS; Rif ^r FUDR ^r	This study
BER-185	638R Δ tdk Δ yifB; Rif ^r FUDR ^r	This study
BER-186	638R Δ tdk Δ uroS Δ yifB; Rif ^r FUDR ^r	This study
BER-201	BER-183 carrying pFD1045; Rif ^r FUDR ^r Erm ^r	This study
BER-202	BER-184 carrying pFD1274; Rif ^r FUDR ^r Erm ^r	This study
BER-203	BER-186 carrying pFD1274; Rif ^r FUDR ^r Erm ^r	This study
BER-204	BER-185 carrying pFD1272; Rif ^r FUDR ^r Erm ^r	This study
BER-208	BER-183/pNBU2- <i>bla-ermG</i> ; Rif ^r FUDR ^r Erm ^r	This study
BER-210	BER-184/pNBU2- <i>bla-tetQ</i> ; Rif ^r FUDR ^r Tet ^r	This study
BER-212	BER-184 carrying pFD1276; Rif ^r FUDR ^r Erm ^r	This study
BER-217	BER-186/pNBU2- <i>bla-tetQ</i> ; Rif ^r FUDR ^r Tet ^r	This study
BER-223	BER-183/pNBU2- <i>bla-tetQ</i> ; Rif ^r FUDR ^r Tet ^r	This study
BER-226	BER-185/pNBU2- <i>bla-tetQ</i> ; Rif ^r FUDR ^r Tet ^r	This study
BER-234	BER-184 carrying pER-323; Rif ^r FUDR ^r Erm ^r	This study
BER-236	BER-184 carrying pER-320; Rif ^r FUDR ^r Erm ^r	This study
BER-237	BER-183 carrying pFD340; Rif ^r FUDR ^r Erm ^r	This study
BER-238	BER-184 carrying pFD340; Rif ^r FUDR ^r Erm ^r	This study
<i>E. coli</i>		
DH10B	Cloning host strain	Invitrogen
HB101::RK231	HB101 containing RK231; (Km ^r) (Tet ^r) (Str ^r)	55
S17-1 λ pir	Strain with the RK2 <i>tra</i> genes for conjugative transfer integrated into the chromosome (RP4-2-Tc::Mu-Km::Tn7 Pro Res ⁻ Mod ⁺ Tp ^r Sm ^r) (λ pir lysogen)	56
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ (DE3)	Novagen
EC308	BL21(DE3) carrying pER-307; Km ^r	This study
SASZ31	F ⁻ <i>hisG1 xyl-7 hemD31 metE46 mobA2 ΔargH1 rplL9(L?) thiE1</i> ; (Str ^r)	33 ^b
EC326 (SASZ31Rif)	SASZ31 spontaneous rifampin-resistant isolate	This study
EC333	SASZ31Rif carrying pFD340; (Str ^r) (Amp ^r) (Rif ^r)	This study
EC334	SASZ31Rif carrying pER-320; (Str ^r) (Amp ^r) (Rif ^r)	This study
EC335	SASZ31Rif carrying pER-323; (Str ^r) (Amp ^r) (Rif ^r)	This study
EC336	SASZ31Rif carrying pER-325; (Str ^r) (Amp ^r) (Rif ^r)	This study
EC339	SASZ31Rif carrying pER-337; (Str ^r) (Amp ^r) (Rif ^r)	This study
Plasmids		
pExchange-tdk	Derivative of pKNOCK- <i>bla-ermG</i> carrying a cloned <i>tdk</i> gene for counterselection; (Amp ^r) Erm ^r	36
pNBU2- <i>bla-ermG</i>	NBU2 integrase (<i>intN2</i>)-based genomic insertion vector derived from pKNOCK- <i>bla-ermG</i> inserts into the NBU2 <i>att1</i> or <i>att2</i> site of tRNA ^{Ser} ; (Amp ^r) Erm ^r	36
pNBU2- <i>bla-tetQ</i>	NBU2 integrase (<i>intN2</i>)-based genomic insertion vector derived from pKNOCK- <i>bla-tetQ</i> inserts into NBU2 the <i>att1</i> or <i>att2</i> site of tRNA ^{Ser} ; (Amp ^r) Tet ^r	21
pET26b(+)	Expression vector with T7 promoter; <i>lacI</i> ; pBR322 replicon; Km ^r	Novagen
pFD288	<i>Bacteroides-E. coli</i> shuttle vector; <i>oriT</i> ; pUC19::pBI143 chimera; (Sp ^r) Erm ^r	34
pFD340	<i>Bacteroides-E. coli</i> expression shuttle vector; (Amp ^r) Erm ^r	39
pFD516	Suicide vector derived from deletion of pBI143 in pFD288; (TetX) (Sp ^r) Erm ^r	34
pFD1045	Shuttle vector derived from pFD340 containing the starch maltose- and oxygen-inducible promoter of the <i>osu</i> operon; (Amp ^r) Erm ^r	57
pFD1241	1,669-bp DNA fragment containing a 528-bp deletion of the <i>tdk</i> gene cloned into the Sall/EcoRI sites of pFD516; (Sp ^r) Erm ^r	This study
pFD1265	3,810-bp DNA fragment containing an internal deletion of the <i>yifB</i> gene cloned into the XbaI/PstI sites of pExchange- <i>tdk</i> ; (Amp ^r) Erm ^r	This study
pFD1272	1,518-bp DNA fragment containing the promoterless <i>yifB</i> gene cloned into the BamHI and SacI sites of pFD340; (Amp ^r) Erm ^r	This study
pFD1274	987-bp DNA fragment containing the promoterless <i>uroS</i> gene cloned into the BamHI and SacI sites of pFD1045; (Amp ^r) Erm ^r	This study
pFD1276	2,601-bp DNA fragment containing entire <i>uroS</i> operon (BF638R_0059–BF638R_0061) cloned into the BamHI/SacI sites of pFD288; (Sp ^r) Erm ^r	This study
pER-302	3,210-bp DNA fragment containing the Δ uroS construct cloned into the XbaI/Sall sites of pExchange- <i>tdk</i> ; (Amp ^r) Erm ^r	This study

(Continued on next page)

TABLE 1 (Continued)

Strain or plasmid	Description or genotype ^a	Reference or source
pER-307	845-bp <i>uroS</i> DNA fragment cloned in frame into the NdeI and XhoI sites of pET26b(+) to construct a UroS fusion recombinant protein with a C-terminal 6×His tag; (Km ^r)	This study
pER-320	987-bp DNA fragment containing the promoterless <i>uroS</i> gene cloned into the BamHI and SacI sites of pFD340; (Amp ^r) Erm ^r	This study
pER-323	854-bp DNA fragment containing the <i>E. coli uroS</i> ORF with 21 bp of the <i>B. fragilis ahpC</i> RBS region placed immediately upstream of the <i>uroS</i> ATG codon (Ec- <i>uroS</i> _{BFRBS}) cloned into the BamHI/SacI sites of pFD340; (Amp ^r) Erm ^r	This study
pER-325	872-bp DNA fragment of the <i>E. coli</i> native <i>uroS</i> gene including 49 bp upstream of the ATG codon cloned into the BamHI/SacI sites of pFD340; (Amp ^r) Erm ^r	This study
pER-337	942-bp DNA fragment containing the <i>B. fragilis uroS</i> gene with 57 bp of the <i>E. coli</i> RBS region placed immediately upstream of the <i>uroS</i> ATG codon (Bf- <i>uroS</i> _{ECRBS}) cloned into the BamHI/SacI sites of pFD340; (Amp ^r) Erm ^r	This study

^aErm^r, erythromycin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance; FUDR^r, 5-fluor-2'-deoxyuridine resistance; Amp^r, ampicillin resistance; Sp^r, spectinomycin resistance; Str^r, streptomycin resistance; Km^r, kanamycin resistance. Parentheses indicate antibiotic resistance expression in *E. coli*.

^bThe SASZ31 strain was obtained from the *E. coli* Genetic Stock Center at Yale University.

Construction of the $\Delta uroS \Delta yifB$ mutant was carried out by conjugating pFD1265, described below, from *E. coli* S17-1 λ pir into BER-184 to obtain $\Delta uroS \Delta yifB$ strain BER-186.

Deletion of the *yifB* gene. A 1,923-bp DNA fragment containing the upstream N-terminal region and a 1,887-bp DNA fragment containing the downstream C-terminal region were amplified by PCR using the primer sets 4364-NT-XbaI-FOR/4364-NT-BamHI-REV and 4364-CT-BamHI-FOR/4364-CT-PstI-REV, respectively. The N-terminal fragment was cloned into the XbaI/BamHI sites of the pExchange-*tdk* vector, followed by the insertion of the C-terminal fragment into the BamHI/PstI sites to construct a 3,810-bp fragment containing an internal deletion of 1,210 bp from the *yifB* gene. The new construct, pFD1265, was mobilized from *E. coli* S17-1 λ pir into BER-183 by biparental mating as described above. Selection for FUDR resistance and erythromycin sensitivity was performed as described above. The deletion of the *yifB* gene by double-crossover genetic exchange in the BER-185 strain was confirmed by PCR analysis using primers 4364_mutcheck-FOR and 4364_mutcheck-REV.

Genetic complementation of the $\Delta uroS$ mutant strain. A 987-bp DNA fragment containing the *uroS* ORF plus 46 bp upstream of the ATG start codon and 179 bp downstream of the stop codon was amplified by PCR from the *B. fragilis* 638R chromosome using primers UroScpmt-BamHI-F and UroScpmt-SalSac-R. The promoterless amplified fragment was cloned into the BamHI and SacI sites of pFD1045. The new construct, pFD1274, was mobilized from *E. coli* DH10B into the BER-184 or BER-186 strain by triparental mating as described above, to make BER-202 and BER-203, respectively. The 987-bp DNA fragment containing the promoterless *uroS* gene was also cloned into the BamHI/SacI sites of pFD340 to construct pER-320. The new plasmid, pER-320, was mobilized from *E. coli* S17-1 λ pir into BER-184 by biparental mating to obtain the BER-236 strain. A 2,601-bp DNA fragment containing the entire *uroS* polycistronic operon was amplified by PCR using primers UroS_operon-FOR and UroS_operon-REV and cloned into the BamHI/SacI sites of pFD288. The new construct, pFD1276, was mobilized from *E. coli* DH10B into BER-184 by triparental mating to obtain BER-212.

For genetic complementation of the *B. fragilis uroS* deletion with the *E. coli uroS* gene, an 854-bp DNA fragment was amplified by PCR from the *E. coli* DH10 chromosome (GenBank accession number [ACB04829](#)) using primers EcUroS-BamHI-FOR and EcUroS-SacI-REV. The oligonucleotide primer EcUroS-BamHI-FOR was designed to place the ribosome-binding site (RBS) of the *B. fragilis ahpC* gene (37, 38) immediately upstream of the *E. coli uroS* ATG codon. This procedure was carried out to replace the *E. coli* RBS region and insert a native *B. fragilis* RBS chromosomal region to optimize the translation of the *E. coli uroS* gene in *B. fragilis*. This DNA fragment (Ec-*uroS*_{BFRBS}) was cloned into the BamHI/SacI sites of pFD340 to construct pER-323. The new construct was mobilized from *E. coli* S17-1 λ pir into the BER-184 strain by biparental mating to obtain the BER-234 strain. The expression of the *uroS* gene is driven by the constitutive IS4351 promoter from the original expression vector pFD340 (39). Promoter sequences located within the insertion sequence (IS) element also function in *E. coli* (39).

For genetic complementation of the *E. coli* SASZ31 *uroS*-deficient strain with the *B. fragilis uroS* gene, 57 bp of the *E. coli* RBS region was placed immediately upstream of the *B. fragilis uroS* ATG codon by PCR amplification from the pER-307 plasmid using the primers pER-307-BamHI-FOR and pER-307-SacI-REV. The amplified 942-bp DNA fragment (Bf-*uroS*_{ECRBS}) was cloned into the BamHI/SacI sites of pFD340. The new construct, pER-337, was mobilized from *E. coli* S17-1 λ pir into SASZ31Rif (EC326) to obtain the *E. coli* EC339 strain. pFD340, pER-320, and pER-323 were also mobilized into the SASZ31Rif (EC326) strain to obtain EC333, EC334, and EC335, respectively. As a control, the native *E. coli uroS* gene containing 49 bp upstream of the ATG codon was amplified by PCR from *E. coli* DH10B using primers EcUroS-NT-FOR and EcUroS-SacI-REV. The 872-bp DNA fragment was cloned into the BamHI/SacI sites of pFD340. The new construct, pER-325, was mobilized from *E. coli* S17-1 λ pir into *E. coli* SASZ31Rif to obtain the *E. coli* EC336 strain.

Genetic complementation of *yifB*. A 1,518-bp DNA fragment containing the *yifB* gene ORF plus 38 bp upstream of the ATG start codon was amplified from the *B. fragilis* 638R chromosome using the primers 4364-Pless-NT-FOR and 4364-Pless-CT-REV. The 1,518-bp DNA fragment containing the promot-

erless *yifB* gene was cloned into the BamHI and SacI sites of the *Bacteroides* expression vector pFD340. The new construct, pFD1272, was mobilized into BER-185 by triparental mating as described above, to make BER-204.

Growth of Δ uroS and Δ yifB mutants and determination of forward and reverse chelate activities. Bacteria were grown on DM containing 5 μ g/ml heme plus 400 μ M BPS or medium containing 5 μ g/ml protoporphyrin IX plus 100 μ M ammonium ferrous sulfate. Media were inoculated with a 1:50 dilution from a culture grown overnight in BHIS medium. Growth was determined by measuring the optical density at 550 nm (OD_{550}) at time points. Bacteria were grown for 24 h anaerobically at 37°C. Bacterial cultures were centrifuged at $12,000 \times g$ for 10 min at 4°C. The pellets were washed twice with phosphate-buffered saline (PBS) (10 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , 145 mM NaCl, 2 mM KCl [pH 7.4]) and centrifuged as described above. The pellets were frozen at $-70^\circ C$ and submitted to the Iron and Heme Core facility, Department of Internal Medicine, Division of Hematology, University of Utah Health Sciences, Salt Lake City, UT, to assay for ferrochelatase and dechelatase specific activities in bacterial crude extracts. The ferrochelatase and dechelatase assays were performed as previously described (9), according to the protocols available at the Iron and Heme Core website (<http://cihd.cores.utah.edu/ironheme/#1465838003097-f4fffc70-f824>).

In vivo dual bacterial competitive survival assays. All procedures involving animals were performed according to the guidelines given in the National Research Council's *Guide for the Care and Use of Laboratory Animals*, 8th ed. (40), and approved by the Institutional Animal Care and Use Committee of East Carolina University.

(i) Intra-abdominal infection. The rat tissue cage model of intraperitoneal infection was used as described previously (41–43). First, a perforated sterilized ping-pong ball was surgically implanted into the peritoneal cavity of a 3- to 4-month-old male Sprague-Dawley rat weighing >400 g (purchased from Charles River Laboratories Inc., Raleigh, NC) and allowed to encapsulate for 4 to 5 weeks. pNBU2-*bla-ermG* or pNBU2-*bla-tetQ* was conjugated into bacterial strains to introduce erythromycin or tetracycline antibiotic resistance markers for bacterial selective isolation and colony counting from mixed infections. Dual bacterial competitive assays were performed as previously described (43). In brief, cultures grown overnight in BHIS medium were diluted in PBS and mixed in a 1:1 ratio of the parent strain/pNBU2-*ermG* (BER-208) to the Δ uroS/pNBU2-*bla-tetQ* (BER-210) or Δ uroS Δ yifB/pNBU2-*bla-tetQ* (BER-217) strain for a total of approximately 1×10^5 CFU/ml of each strain as a standard inoculum. Dual colonization with the parent strain carrying pNBU2-*bla-ermG* (BER-208) versus the parent strain carrying pNBU2-*bla-tetQ* (BER-223) was also performed as a control for possible inherently intrinsic variables when using antibiotic resistance markers for differential CFU quantification of laboratory strains recovered directly from infectious specimens. An aliquot of the inoculum suspension was serially diluted in PBS and plated on erythromycin (10 μ g/ml) or tetracycline (5 μ g/ml) to determine inoculum CFU per milliliter. Four milliliters of the inoculum was 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 medium containing rifampin (20 μ g/ml), gentamicin (100 μ g/ml), and FUDR (200 μ g/ml) plus erythromycin (10 μ g/ml) or tetracycline (5 μ g/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 the CFU per milliliter of tissue cage serous fluid. The limit of detection was 1×10^1 CFU/ml. Competitive indices were calculated for each rat by dividing the number of surviving mutant cells by the number of surviving parent strain cells. This was then divided by the ratio of the mutant to the parent strain cells in the inoculum input, as previously described (43).

(ii) Intestinal colonization. Specific-pathogen-free (SPF), 6- to 8-week-old, male C57BL/6J mice were purchased from Jackson Laboratories and housed under microisolation conditions (animal biological safety level 2 housing facility). These mice were used for dual colonization with the parent strain/pNBU2-*bla-ermG* (BER-208) versus the Δ uroS/pNBU2-*bla-tetQ* (BER-210) or Δ uroS Δ yifB/pNBU2-*bla-tetQ* (BER-217) strain. According to other established mouse enteric colonization models (44–47), 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 to the water to increase consumption since other reports have indicated occasional refusal of mice to drink water containing antibiotics (44). Antibiotic treatment was carried out for 7 days and subsequently replaced with sterile drinking water. Two days and four days following the 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 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 cecum content specimens were weighed, serially diluted in sterile PBS, and plated on rifampin (20 μ g/ml), gentamicin (100 μ g/ml), and FUDR (200 μ g/ml) plus erythromycin (10 μ g/ml) or tetracycline (5 μ g/ml), as described above. For dual colonization with BER-180 versus BER-51 strains, cecum content dilutions were plated onto medium containing rifampin (20 μ g/ml) and gentamicin (100 μ g/ml) plus fusidic acid (12 μ g/ml) or tetracycline (5 μ g/ml). The CFU counts were normalized to CFU per gram of cecum content.

Statistical analysis. The data were analyzed with GraphPad Prism software version 8.3.0 using an unpaired, parametric, two-tailed *t* test. Student's *t* test at a 95% confidence interval was employed for comparisons of the two groups. A *P* value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.01 MB.

SUPPLEMENTAL FILE 2, PDF file, 1.5 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 4, PDF file, 4.5 MB.
SUPPLEMENTAL FILE 5, PDF file, 0.2 MB.
SUPPLEMENTAL FILE 6, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 7, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 8, PDF file, 0.1 MB.

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