Deficiency of the ferrous iron transporter FeoAB is linked with metronidazole resistance in Bacteroides fragilis

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Background: Metronidazole is the most commonly used antimicrobial for Bacteroides fragilis infections and is recommended for prophylaxis of colorectal surgery. Metronidazole resistance is increasing and the mechanisms of resistance are not clear.

Methods: A transposon mutant library was generated in B. fragilis 638R (BF638R) to identify the genetic loci associated with resistance to metronidazole.

Results: Thirty-two independently isolated metronidazole-resistant mutants had a transposon insertion in BF638R_1421 that encodes the ferrous transport fusion protein (feoAB). Deletion of feoAB resulted in a 10-fold increased MIC of metronidazole for the strain. The metronidazole MIC for the feoAB mutant was similar to that for the parent strain when grown on media supplemented with excess iron, suggesting that the increase seen in the MIC of metronidazole was due to reduced cellular iron transport in the feoAB mutant. The furA gene repressed feoAB transcription in an iron-dependent manner and disruption of furA resulted in constitutive transcription of feoAB, regardless of whether or not iron was present. However, disruption of feoAB also diminished the capacity of BF638R to grow in a mouse intraperitoneal abscess model, suggesting that inorganic ferrous iron assimilation is essential for B. fragilis survival in vivo.

Conclusions: Selection for feoAB mutations as a result of metronidazole treatment will disable the pathogenic potential of B. fragilis and could contribute to the clinical efficacy of metronidazole. While mutations in feoAB are probably not a direct cause of clinical resistance, this study provides a key insight into intracellular metronidazole activity and the link with intracellular iron homeostasis.

Keywords: iron transport, anaerobic bacteria, antimicrobial resistance, transposon mutant

Introduction

Bacteroides fragilis is an anaerobic commensal that, along with other Bacteroides species, performs vital functions in the human gastrointestinal tract, including energy harvesting, bile acid recycling, development of the host immune system1,2 and providing protection from inflammation by inducing a subset of T cells that secrete the anti-inflammatory cytokine interleukin-10.2 However, if B. fragilis moves out of its niche in the gut, it can cause serious, and potentially fatal, infections in humans; it is the most frequently isolated anaerobic bacterium from patients with deep tissue infections, intra-abdominal infections, bacteremia, abscesses and endocarditis.1,3,4

Metronidazole is the most commonly used drug worldwide to treat infections involving B. fragilis5,6 and has been the treatment of choice for surgical prophylaxis (e.g. for colorectal surgery) for almost 40 years.7,8 Levels of metronidazole resistance in Bacteroides species had remained remarkably low, but recently there has been a gradual trend in increased resistance that may relate to increasing use of metronidazole for other infections. For example, metronidazole use has widened to include treatment of Clostridium difficile infection9 and eradication regimens for Helicobacter pylori.10 A worrying development is the report of multidrug-resistant isolates and treatment failure.11–13 Elucidating the mechanisms by which B. fragilis can gain metronidazole resistance provides important information about the
potential spread of resistance as well as options for drug development.

Metronidazole is a prodrug and its cytotoxicity depends on the reduction of the nitro group to a toxic nitro anion radical, which may cause DNA damage and/or indirectly interfere with the redox system of the microbe. The redox environment of anaerobic bacteria facilitates the activation of the prodrug to its toxic form. Reduction of metronidazole both activates it and creates a concentration gradient, driving further drug uptake and therefore increases the concentration of the toxic intermediates. Understanding the mechanisms of activation and action of metronidazole, as well as resistance to metronidazole, has been the subject of study in the past several years. This has included detailed investigation of metronidazole activity in Helicobacter species and protozoan organisms in addition to anaerobic bacteria. Despite this, the exact mode of transforming the non-toxic prodrug to the toxic free-radical intermediate is not completely clear, although the redox system of the target microbe is known to be involved. The very low redox potential of metronidazole limits possible electron donors. In anaerobes, ferrodoxin-like Fe-S proteins have a lower redox potential; thus, the pyruvate:ferredoxin oxidoreductase (PFOR) system has formed the basis for current models of metronidazole activation. Evidence for this model includes observed increases in metronidazole resistance with down-regulation of pyruvate oxidoreductase via metabolic shift to the conversion of pyruvate into lactate via dehydrogenase rather than pyruvate oxidoreductase. More recent data in Trichomonas suggest a second, non-PFOR, flavin-based mechanism of metronidazole activation, in which the flavin enzyme thioredoxin reductase reduces metronidazole and is not dependent on the iron status of the cell. Reactive metabolites of metronidazole can then form covalent adducts with thioredoxin reductase and other proteins that are known or assumed to be associated with thioredoxin-mediated redox regulation (ribonucleotide reductase, thioredoxin peroxidase and cytosolic malate dehydrogenase).

Metronidazole resistance mechanisms in B. fragilis are also complex. There is a requirement for activation and any alteration in metabolic activity that prevents or reduces activation can increase resistance. Also, antimicrobial action can cause DNA damage and mutations that result in improved DNA repair can increase resistance. Additionally, some B. fragilis strains produce a 5-nitroimidazole reductase (Nim) that converts the nitro group of metronidazole into the non-toxic amine derivative. Several different nim genes (nimA–H) have been reported for B. fragilis and a novel nim gene (nimJ) that is not recognized by the ‘universal’ nim primers was reported recently. It is possible that reported ‘nim-negative’ clinical isolates contain a functional nim gene that is unrecognizable by the published ‘universal’ primers, but it is also clear that metronidazole resistance can be nim-based. Our research and that of others indicates that non-nim-based mechanisms are important in clinical levels of resistance to metronidazole. For example, nimJ and nimE, from two different metronidazole-resistant clinical isolates, could confer increased metronidazole to BF638R, but not to the MIC levels seen in the clinical isolate. Several nim-negative, metronidazole-resistant B. fragilis strains have been reported and metronidazole resistance is also inducible in the metronidazole-susceptible type strain B. fragilis NCTC 9343. If mutated, in addition to the PFOR system, genes involved in the regulation of sugar catabolism, resistance–nodulation–division family Bacteroides multidrug efflux (bme) pumps and DNA repair can impact Bacteroides metronidazole susceptibility. This study demonstrates that mutation in an iron transport protein, feoAB, generated by transposition-mediated gene disruption, enhances metronidazole resistance and provides evidence of the key role of intracellular iron in susceptibility to metronidazole.

Methods

Strains and culture conditions
All feoAB constructs were derived from BF638R (Table 1). Primers used in the construction of specific mutants are listed in Table S1 (available as Supplementary data at JAC Online). The specific construct used in each experiment is described below. Bacterial strains were grown at 37°C as described previously using brain heart infusion (BHI) media supplemented with 15 mg/L haemin (BHI/S) for B. fragilis isolates in H. M. Wexler’s laboratory (Anabaena Systems, Morgan Hill, CA, USA) or BHI supplemented with 1 g/L L-cysteine, 5 mg/L haemin, 1 mg/L resazurin and 20 mL/L 10% NaHCO3 (BHI-ER) in E. R. Rocha’s laboratory and Luria–Bertani agar or broth (Sigma) for Escherichia coli DH10B. The concentration of haemin used in different laboratories is above the minimum required for B. fragilis growth. For some experiments, bacteria were grown in semi-defined medium (SDM) as described previously. Addition of 50 μM 2,2′-bipyridyl was used to restrict iron availability in SDM. Ferrous sulphate at 100 μM was added for high-iron growth conditions.

Bacteroides isolates were incubated in anaerobic jars (AnaXomat, 5% CO2, 5% H2 and 90% N2) or in an anaerobic chamber (10% CO2, 10% H2 and 80% N2). E. coli was incubated in ambient air at 37°C. Ampicillin (50 mg/L), erythromycin (10 mg/L), rifampicin (10 mg/L), gentamicin (25 mg/L) and kanamycin (40 mg/L) were used for selection as indicated.

MIC determination
MICs were determined using Etest strips (bioMérieux, Durham, NC, USA).

Molecular methods
DNA preparation, restriction digests, gel electrophoresis and analysis were done as described previously.

Generation of metronidazole-resistant transposon mutants
A BF638R transposon mutant pool (50000 isolates) was generated using the mariner transposon-based vector pSAM-Br as described previously and stored at −80°C. For the isolation of metronidazole-resistant mutants, 50 μL from the transposon mutant pool (2.9 × 106 cfu) was plated onto BHI agar plates containing 0.5 mg and 1 mg/L metronidazole, respectively (MIC of metronidazole for BF638R is ~0.2 mg/L). The plates were incubated at 37°C for 2–3 days under anaerobic conditions. Following incubation, 40–50 colonies appeared on each plate and 32 (8 randomly selected from each plate) were chosen for further study.

Identification of transposon-disrupted genes
The transposon-disrupted genes were identified by semi-random primer PCR as described previously.

Deletion of BF638R_1421 in BF638R
Approximately 500 bp of upstream and downstream sequence of BF638R_1421 was PCR amplified (upstream primers, 638R_1421 upstream primers, 638R_1421 up...
UpF/638R_1421 ermF JoinR; downstream primers, 638R_1421 ermF Join F/638R_1421 UpR, Table S1) fused to an erm(F) cassette amplified with primers 638R_1421 ermF and 638R_1421 ermF (Table S1) by overlapping PCR and cloned into the suicide vector pGB909[d38R_1421 Up-erm(F)F-Do] to generate pGB909[d38R_1421 Up-erm(F)F-Do]. The generated suicide vector was used to replace BF638R_1421 with erm(F) in BF638R by homologous recombination and the deletion was verified by PCR with primers 638R_1421 and 638R_1421 and sequencing.

Two independently derived strains of the resulting erm(F) mutants (biological replicates) were chosen for further study (BF638R::fdeoAB-A and BF638R::fdeoAB-B). Also, two separate transposon mutants with mutations in the fdeoA operon region (BF638R::fdeoA-MET14) and fdeoB region (BF638R::fdeoB-MET1) of BF638R_1421, respectively, were used in MIC determinations.

**Isolation of a fdeoAB::pFD516 revertant to the wild-type fdeoAB gene**

The BER-1 strain was grown in BHIS-ER broth through five consecutive passages in the absence of erythromycin. An aliquot of the culture was then used to inoculate iron-limiting SDM supplemented with haemin as described previously to allow for the enrichment of the culture with revertants of single cross-over back to wild-type genotype. After a second passage in the same fresh medium, the culture was plated onto BHIS-ER agar. Fifty colonies were picked up and spotted onto two sets of BHIS-ER plates (one plate containing rifamycin, gentamicin and erythromycin and a second plate containing rifamycin and gentamicin only). Erythromycin-susceptible colonies were selected and the reversion of the fdeoAB allele to wild-type was confirmed by PCR amplification with primers FdeoAB-operon-1F and FdeoAB-operon-1R (Table S1) and sequencing.

**Construction of fdeoAB insertion mutant**

A 725 bp internal N-terminus fragment of BF638R_1421 was PCR amplified with primers FdeoAB1-Forward and FdeoAB1-Reverse (Table S1) and cloned into the pGEM-T vector (Promega, Madison, WI, USA). An SpH/SacI fragment containing the fdeoA internal region was then cloned into the SpH/SacI sites of the suicide vector pFD516. The new construct, pER-1, was mobilized from E. coli DH10B into BF638R by a triparental filter mating protocol. Transconjugants were selected on BHIS agar containing 20 mg/L rifamycin, 100 mg/L gentamicin and 10 mg/L erythromycin. PCR analysis was used to confirm the insertion of pER-1 into the BF638R chromosome. A transconjugant, BER-1, containing the fdeoAB::pFD516 construct inserted into the BF638R chromosome was selected for further studies.

**Construction of a fdeoAB::β-xylosidase (xylB) transcripational fusion reporter gene**

A furA deletion mutant was constructed as described previously. A fdeoAB::β-xylosidase reporter gene in a fdeoAB mutant background was constructed by cloning a 1.3 kb promoterless β-xylosidase/a-arabinosidase bifunctional reporter gene into the EcoRI site of pER-1. The new construct, pER-4, was mobilized into the BF638R parent strain and the furA deletion mutant, BER-2, as described above. The new constructs,
BER-17 (BF638R feoAB::xylB) and BER-18 (BF638R ΔfurA feoAB::xylB), were used for transcriptional analysis of the feoAB gene under iron-replete and iron-limiting conditions.

**Enzyme assays**

β-Xylosidase assays in crude extracts of bacteria grown in SDM under iron-replete and iron-limiting conditions (bipyrindiamide used to limit iron) were performed as described previously in detail.28

**Construction of a feoAB deletion mutant strain**

To construct the feoAB deletion mutant, a 1.876-kb internal DNA fragment from feoAB was deleted and replaced by the tetracycline resistance gene tetQ. Briefly, a 1.615-kb chromosomal fragment upstream of BF638R feoAB (BF638R_1421), including the first 70 nt within the N-terminus region, was amplified by PCR using primers FeoAB-BamHI-Forward and FeoAB-BglII-Reverse (Table S1). The amplified DNA fragment was cloned into the unique BamHI site of the E. coli: Bacteroides shuttle suicide vector pFD51652 containing a 2.6-kb tetracycline tetQ resistance cassette in the BamHI/SstI site. Subsequently, a 1.791-kb fragment downstream from feoAB, containing the last 546 nt of the C-terminus region, was amplified by PCR using primers FeoAB-SstI-Forward and FeoAB-SstI-Reverse (Table S1). The amplified fragment was cloned into the unique SstI site of the new construct pER-66. The plasmid pER-66, containing the ΔfeoAB::tetQ construct, was mobilized from E. coli DH10B into BF638R by triparental filter mating protocols as described above. Transconjugants were selected on BHIS-ER agar containing 20 mg/L rifamycin, 100 mg/L gentamicin, 5 mg/L tetracycline and 10 mg/L erythromycin. The new strain, BER-125 (BF638R feoAB::tetQ), was selected for further studies.

**Construction of a complementation vector for BF638R_1421**

The B. fragilis expression vector pLYL10115 was used for cloning the feoAB gene. To construct the complementation vector with a strong promoter, 102 nt of the promoter for the polysaccharide H (PSH) gene (i.e. upstream of BF9343_3376) was amplified using primers 9343 PmmVF PslI/638R_1421 P JoinR (Table S1). BF638R_1421 was amplified using primers 638R_1421 P JoinF/638R_1421 SphIR (Table S1). The two amplicons were stitched by overlapping PCR. The resulting PCR product was digested with PstI/SphI and ligated into PstI/SphI-digested pCYL101 and cloned into E. coli Mach1 (NEB). The resulting vector, pLYL101-BF638R_1421, was mobilized into BF638R by triparental mating of E. coli/pLYL101:PSH promoter-BF638R_1421, BF638R and E. coli:pRK231 helper as described previously.23

A second complementation vector for feoAB was separately constructed in the BER-51 strain. Briefly, a 2833 bp DNA fragment containing 225 bp upstream of the feoAB ATG start codon and 1225 bp downstream of the stop codon was PCR amplified from BF638R using primers FeoAB-operon-2Forward and FeoAB-operon-2Reverse, designed to incorporate BamHI and BglII sites, respectively (Table S1). The PCR-amplified fragment was cloned into the BamHI site of the shuttle vector pFD282.22 Automated nucleotide sequencing was used to confirm the native feoAB gene in the new construct pER-191. pER-191 was mobilized into BER-51 by triparental mating as described above. Transconjugants were selected on BHIS-ER agar containing 20 mg/L rifamycin, 100 mg/L gentamicin, 5 mg/L tetracycline and 10 mg/L erythromycin. The new strain, BER-125 (BF638R feoAB::tetQ feoAB2), was used for genetic complementation analysis.

**Measurement of transcription levels by quantitative (q) RT–PCR**

Transcription levels of selected genes were determined using qRT–PCR. Total RNA for qRT–PCR was prepared from early to mid-log growth strains (OD600 0.6–0.8) grown in BHIS using the RNAeasy mini kit with RNAProtect (Qiagen, CA, USA). Contaminating DNA was removed with the RNase-free DNAase Set (Qiagen, CA, USA). qRT–PCR studies were done using SYBR Green RT–PCR Mix (Applied Biosciences, CA, USA or Bio-Rad Laboratories) and the StepOnePlus instrument (Applied Biosciences, CA, USA) or the iCycler (Bio-Rad Laboratories). The primers used for qRT–PCR analysis are listed in Table S2 (available as Supplementary data at JAC Online). The comparative CT (ΔΔCT) method was used to determine the relative transcription levels using 16S RNA as the endogenous control. The analysis program is part of the StepOne software.

**Global transcriptome analysis**

Bacteria were grown in BHIS to mid-log growth (OD600 0.8–1) and RNA was prepared from cells using the Qiagen RNeasy Kit (Qiagen, CA, USA) according to the manufacturer’s instructions. rRNA was removed using the Ambion MicroBioExpress Kit (Life Technologies). cDNA was prepared using the Invitrogen Superscript Kit (Life Technologies) and quantified by RNA-seq analysis (Otogenetics, Norcross, CA, USA). RNA-Seq files were analysed using the Lasergene Genomics Suite (DNASTAR, Madison, WI, USA).

**In vitro growth measurements**

In vitro growth of BF638R and BF638R BER-1 feoAB were measured in defined minimal medium and in BHI broth (37 g/L, Oxoid) supplemented with haemin (0.5 mg/L) and cysteine hydrochloride (0.5 g/L). The culture medium also contained appropriate antibiotic for selection purposes. At least two growth rate experiments were carried out for each strain of BF638R analysed in both defined medium and supplemented BHI, with total viable counts taken at various timepoints.

**In vivo growth measurements**

In vivo growth of BF638R, BF638R/pDF340 and BF638R BER-1 feoAB was examined using a mouse chamber model of peritoneal infection as described previously.37,38 Briefly, two chambers containing live bacterial suspension, bound at either end with 0.45 μm pore nitrocellulose membrane filters (Millipore), which allow the influx of soluble host factors but not mouse cells and preclude the efflux of bacterial cells, were implanted in the mouse peritoneal cavity after surgical incision; the surgical wound was then sutured. In this model, bacteria are grown in vivo without causing peritoneal infection. After the required incubation time, mice were sacrificed (at least two mice per timepoint), the chambers removed and the total viable count determined by serial dilution and agar plating. The experiments were licensed by the Department of Health, Social Services and Public Safety Program and Personal Licenses and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. B. fragilis pDF340 was used as a positive control for BF638R BER-1.

**Results**

Metronidazole resistance is linked to disruption of a putative iron transport gene feoAB (BF638R_1421)

Of the metronidazole-resistant transposon mutants generated using the transposon vector mutant,30 32 were selected at random and restreaked onto BHI agar containing 1 mg/mL metronidazole. All of these mutants had transposon insertions in BF638R_1421, a putative transmembrane ferrous transport fusion protein gene (Figure 1). None of the 32 mutants had unique

![Image](https://academic.oup.com/jac/article-abstract/69/10/2634/689463/16)
Table 2. Metronidazole MICs in supplemented BHIS with and without added iron

<table>
<thead>
<tr>
<th>Strain/genotype</th>
<th>without added Fe</th>
<th>with added Fe</th>
</tr>
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<tbody>
<tr>
<td>BF638R</td>
<td>0.19 ± 0.05</td>
<td>0.19</td>
</tr>
<tr>
<td>BF638R ΔfeoAB</td>
<td>1.4 ± 0.18</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>BER-2</td>
<td>0.19</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>BER-1</td>
<td>1.5</td>
<td>0.25</td>
</tr>
<tr>
<td>BER-51</td>
<td>1.73 ± 0.35</td>
<td>0.5</td>
</tr>
<tr>
<td>BER-54</td>
<td>0.18 ± 0.09</td>
<td>0.25</td>
</tr>
<tr>
<td>BER-125</td>
<td>0.15 ± 0.04</td>
<td>0.25</td>
</tr>
<tr>
<td>BF638R ΔfeoA-MET14</td>
<td>0.75</td>
<td>ND</td>
</tr>
<tr>
<td>BF638R ΔfeoB-Met-1</td>
<td>0.75</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.

Assays repeated at least three times.

BF638R wild-type strain (0.19 mg/L). Metronidazole MICs for BF638R ΔfeoAB without added ferrous sulphate were 1.4 ± 0.18 mg/L and MICs with added ferrous sulphate were 0.26 ± 0.04 mg/L (Table 2).

**BF638R_1421 encodes a FeoAB transport protein**

The predicted amino acid sequence of BF638R_1421 (827 amino acids) was examined for conserved domains at the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The protein sequence of BF638R_1421 contained regions matching all of the conserved domains of the FeoA and FeoB proteins of E. coli (Figure 1, upper panel).

Thus, BF638R_1421 is a FeoA–FeoB hybrid protein and is probably involved in ferrous iron uptake.

**Growth of BER-1 (BF638R feoAB::pFD516) is attenuated in medium without supplemental ferrous iron compared with BF638R/pFD340**

The doubling time of BER-1 in vitro is 8.59 h in defined medium and 2.64 h in BHIS-ER, which is 2–4-fold higher than that of...
BF638R/pDF340 (2.18 h in defined medium and 1.03 h in BHIS) (Table 3). Moreover, the growth of BER-1 is reduced in SDM under low-iron conditions but not under high-iron conditions compared with the parent strain (E. R. Rocha and C. J. Smith, unpublished data). These results validate the importance of the FeoAB uptake system in B. fragilis and confirm previous studies showing that B. fragilis requires both inorganic iron and haem iron for maximum growth. The growth of the feoAB-deficient strains in BH broth or BHIS agar, although slower than the wild-type, indicates that disruption of BF638R_1421 does not affect the normal physiology of the cells when iron is plentiful, suggesting that other iron uptake mechanisms exist. In contrast, the growth rate of BER-2 (BF638R ∆furA) was similar to wild-type BF638R (Table 3).

Overexpression of feoAB appears to be toxic in B. fragilis feoAB-deficient mutants

BF638R_1421 (feoAB) was cloned into the B. fragilis expression vector pLYL101 and transformed into E. coli; the clone was verified by sequencing. Despite several attempts to introduce it back into BF638R ∆feoAB by triparental mating to complement the deletion, a clone with an intact gene could not be isolated. A few transformants were isolated, but the gene sequence of feoAB on the expression plasmid had been disrupted by sequence rearrangements, suggesting that overexpression of feoAB on pLYL101 is toxic to BF638R.

In contrast, when the native feoAB gene in pER-191 was mobilized into the feoAB deletion mutant BER-51, metronidazole susceptibility in the complemented strain, BER-125, was restored to the levels of the parent strain. Taken together, these data suggest that the transcription level of feoAB needs to be regulated by its own normal regulatory mechanisms and that overexpression seems to be toxic to the cell.

Genes implicated in spontaneous metronidazole resistance were not down-regulated

Earlier studies had demonstrated diminished expression, and in some instances transcription, of several genes in spontaneous metronidazole-resistant mutants, including tpx (thioredoxin peroxidase), bfr (bacterioferritin), fldA (flavodoxin) and nifJ (aka para; pyruvate:flavodoxin oxidoreductase). Therefore, transcription levels of these genes were measured in the metronidazole-resistant BF638R ∆feoAB using qRT-PCR analysis. No changes in transcription levels of tpx (BF638R_2372), bfr (BF638R_3305), fldA (BF638R_2696) and nifJ (BF638R_3194) between BF638R and BF638R ∆feoAB were seen, indicating that diminished transcription of these genes is not responsible for the observed metronidazole resistance of BF638R ∆feoAB.

Transcription levels of typical iron stress-related genes were not increased

A defect in FeoAB might result in lowered iron transport and iron stress. Therefore, transcription levels of iron starvation-related genes in BF638R ∆feoAB were measured. In E. coli, Fe2⁺ starvation induces bfd (ferredoxin) and restricts ftnA (Fe storage) expression. The iron uptake regulator (fur) is also induced during Fe2⁺ starvation in H. pylori. Since FeoAB is an iron transporter, the effect of feoAB disruption on the transcription of genes that are known to be altered during iron starvation was measured. qRT-PCR analysis showed no difference between wild-type BF638R and BF638R ∆feoAB in expression of nifJ (BF638R_3194), furA (BF638R_3276 and BF638R_3026), ftnA (BF638R_2891) and ferredoxin (BF638R_1702) genes (data not shown). Therefore, it appears that disruption of BF638R_1421 does not induce the typical response to iron starvation in BF638R. Thus, it is likely that in an iron-rich medium such as BH, another mechanism for obtaining iron becomes operative and provides adequate iron to avert iron stress but insufficient intracellular iron to confer wild-type levels of metronidazole susceptibility.

Global transcriptome changes between BF638R and BF638R ∆feoAB

Global transcriptome changes between BF638R and BF638R ∆feoAB were determined. RNA-Seq analysis showed down-regulation of several dehydrogenase/oxidoreductase genes in BF638R ∆feoAB. Among them, a putative dehydrogenase/oxidoreductase (BF638R_1133; nifJ) was down-regulated >2-fold in BF638R ∆feoAB compared with BF638R (Table S3, available as Supplementary data at JAC Online). In addition, nuaA – J, genes that code for respiratory complex I, were also down-regulated by >2-fold in BF638R ∆feoAB. The transcription levels of the genes BF638R_1133, BF638R_3326 (rdxA) and two genes, NADH-quinone oxidoreductase chain A and B (nuaA and nuaB), from the nua operon (BF638R_0849 and BF638R_0850) were measured by qRT–PCR analysis in BF638R and BF638R ∆feoAB (biological replicates A and B) and in six independent feoAB-transposon

Table 3. Growth of BF638R and mutants in defined medium and in BHIS-ER medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth in defined medium</th>
<th>Growth in BHIS-ER</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>specific growth rate (h⁻¹)</td>
<td>doubling time (h)</td>
</tr>
<tr>
<td>BF638R</td>
<td>0.66 ± 0.19</td>
<td>1.22 ± 0.28</td>
</tr>
<tr>
<td>BF638R/pFD340</td>
<td>0.32</td>
<td>2.18</td>
</tr>
<tr>
<td>BER-1</td>
<td>0.081</td>
<td>8.59</td>
</tr>
<tr>
<td>BER-2</td>
<td>0.56</td>
<td>1.23</td>
</tr>
</tbody>
</table>

*Mean of three independent growth curves.

*Mean of two independent growth curves.

*Specific growth rate was calculated using Napierian logs and is expressed as h⁻¹.
Transcription analysis of *feoAB* mRNA expression in BF638R parent and *furA* mutant strains grown in semi-defined medium under high-iron (+Fe) and iron-limiting (−Fe) conditions. (a) Real-time RT–PCR relative quantification of *feoAB* mRNA expression in total RNA extracted from cultures in mid-log growth from wild-type BF638R and BF638R ΔfurA (BER-2). (b) β-Xylosidase activity determination in crude extracts of cultures from BF638R *feoABːxylB* (BER-17) and BF638R ΔfurA *feoABːxylB* (BER-18) in mid-log growth.

**Figure 2.**

Mutants. Transcription of BF638R_1133 was consistently ≥2-fold lower in BF638R Δ*feoAB* compared with BF638R, but consistent down-regulation for the other genes was not seen.

**feoAB** transcription is regulated by iron in a Fur-dependent manner

The effect of Fur regulation on *feoAB* mRNA expression was investigated by measuring the levels of *feoAB* mRNA in BF638R and BF638R Δ*furA* (BER-2) by qRT–PCR under high-iron and iron-limiting conditions (Figure 2a). When BF638R was grown under high-iron conditions, repression of *feoAB* expression was observed, which switched to derepression under iron-limiting conditions. In BF638R Δ*furA* (BER-2), however, the iron-mediated repression of *feoAB* transcription was no longer observed, indicating that FeoAB is controlled by Fur. The real-time RT–PCR determination of *feoAB* mRNA expression was confirmed by transcription fusion reporter β-xylosidase gene experiments in crude extracts of cultures from BF638R *feoAB::xylB* (BER-17) and BF638R Δ*furA* *feoAB::xylB* (BER-18) under high-iron conditions compared with the levels of activity obtained under iron-limiting conditions (Figure 2b).

**Growth and survival of *feoAB*- and *furA*-deficient BF638R in vivo**

The abilities of BF638R, BER-1 (BF638R Δ*feoAB*), BF638R/pFD340 and BER-2 (BF638R Δ*furA*) to survive and grow in an intraperitoneal abscess model were compared (Figure 3). Reduction in the number of viable bacteria over time was observed until the number of viable bacteria was below detectable levels of 250 cells/mL after 21 days in the mouse peritoneal cavity. Growth of BER-1 and BER-2 were attenuated in the mouse intraperitoneal abscess model.

**Figure 3.**

Using an unbiased transposon mutagenesis procedure to generate a library of metronidazole-resistant mutants, 32 metronidazole-resistant mutants independently chosen for further study had a mutation in the iron uptake-associated *feoAB* gene. This indicates that mutation of *feoAB* is a favoured selection for evasion of metronidazole toxicity. Therefore, the effect of *feoAB* deletion on susceptibility to metronidazole was studied in more detail.

Although it is possible that the deficiency in *feoAB* might initiate a ‘low-iron stress’ response, altered transcription levels for putative genes involved in iron transport and uptake in the parent strain were not seen. Changes in the global transcriptome of the
The fur system has also been implicated in metronidazole resistance in *H. pylori*. Several induced mutations affecting *H. pylori* Fur’s N-terminal arm increased metronidazole resistance, as did mutations affecting the region between DNA binding and dimerization domains. The authors concluded that *fur* mutations can affect susceptibility to metronidazole by altering the balance among Fur’s several competing activities, affecting expression of genes that control cellular redox potential or elimination of bactericidal metronidazole activation products.

The *feoAB* system plays an important role in ferrous iron uptake and is also important in pathogenic processes in *E. coli* and other pathogenic bacteria, especially under anaerobic/microaerophilic conditions. We found that *feoAB*Δ (BER-1) was attenuated in its ability to grow in an intraperitoneal mouse model. Similarly, *feoB* mutants of *E. coli* and *Salmonella* are attenuated in their ability to colonize mouse intestines. Overexpression of *feoAB* was not tolerated in *BF638R*, suggesting that iron uptake needs to be carefully regulated for optimum cell growth and function of *B. fragilis*. Also, disruption of *feoAB* did not result in growth deficiency in iron-rich media, yet the amount of iron taken up was not sufficient to abrogate the metronidazole resistance seen, further indicating that the precise levels of iron uptake will have profound effects on cell metabolic processes.

Since the ability to assimilate iron is an important virulence factor, it is perhaps unlikely that a defect in *feoAB* is a clinical mechanism for metronidazole resistance. We hypothesize that the metronidazole-induced reduction in virulence contributes to the efficacy of the antimicrobial action of metronidazole in infections and in prophylaxis. We have demonstrated that strains with *feoAB* gene disruptions are selected by metronidazole and that the *feoAB* mutants are disabled with respect to virulence potential. This could also have played a part in the, to date, slow development of metronidazole resistance in *B. fragilis*. The reasons for the increasing rate of metronidazole resistance are still not clear. Increasing horizontal transfer of *nim* genes is a worrying factor, although the contribution of *nim* genes to the high metronidazole MICs seen for clinically resistant isolates is being questioned. In a recent study, no correlation was found between *nim* levels and metronidazole MICs and no evidence was found that Nim proteins protect *B. fragilis* from metronidazole by sequestering the activated antibiotic. We did find modest increases in metronidazole MICs when *nimE* or *nimJ* was introduced into *BF638R* on a multicopy expression plasmid. It is possible that the high metronidazole MICs seen for clinically resistant isolates are due to a combination of resistance factors (both *nim* genes and other factors, including some that may not yet be described). Furthermore, it appears that these factors have the potential to override the heretofore slow rise in metronidazole resistance and, in this context, the growing global evidence of *nim* gene horizontal gene transfer is a particular cause for concern as it may lead to an acceleration in the incidence of metronidazole resistance in the near future. The data presented here indicate that the susceptibility of *B. fragilis* to metronidazole is dependent on the iron status of the cell, in which iron uptake by *feoAB* plays a key role. This supports the hypothesis that an iron-dependent system such as PFOR is the operant mechanism for metronidazole activation in *B. fragilis*. Further analysis of the role of *feoAB*- and *furA*-regulated genes and the effect of iron levels on the organism is needed to fully understand the functioning and significance of all aspects of this metronidazole resistance pathway.

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References
17. Leitsch D, Kolarich D, Binder M et al. Trichomonas vaginalis: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thior edoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. Mol Microbiol 2009; 72: 518–36.


