Transcriptional regulation of the Bacteroides fragilis ferritin gene (ftnA) by redox stress

Edson R. Rocha and C. Jeffrey Smith

This study shows that the iron-storage protein ferritin is a component of the redox-stress response in the obligate anaerobe Bacteroides fragilis. It is up-regulated at transcriptional level under aerobic conditions but constitutively expressed at low levels under anaerobic conditions. Northern hybridization and primer extension analysis revealed that ftnA is transcribed as a monocistronic mRNA of approximately 600 nt. Under reduced anaerobic conditions, ftnA mRNA levels were not dependent on the iron content of the culture medium. Following oxygen exposure ftnA message increased about 10-fold in iron-replete medium compared to a fourfold increase under low-iron conditions. Addition of the oxidant potassium ferricyanide induced expression of ftnA mRNA anaerobically, suggesting that the oxidation of the medium affected expression of ftnA. Two transcription initiation start sites were identified. Both transcripts were expressed constitutively under anaerobic conditions but one promoter was induced by oxidative stress or the addition of the oxidant potassium ferricyanide. The effect of redox stress on ftnA expression was further investigated by addition of diamide, a thiol-oxidizing agent, which induced ftnA mRNA levels anaerobically, suggesting that an unbalanced cellular redox state also affects ftnA expression. Induction by hydrogen peroxide and oxygen was decreased in an oxyR deletion mutant but some oxygen induction still occurred. This strongly suggests that ftnA is regulated by both the peroxide response transcriptional activator, OxyR, and another unidentified oxygen-dependent regulator. Taken together, these data show that ftnA mRNA levels are controlled by both iron and oxidative stress; this coordinated regulation may be important for survival in an adverse aerobic environment.

INTRODUCTION

Iron acquisition occurs widely in the microbial world, but free iron in the presence of oxygen is potentially toxic by generating cell-damaging oxygen radicals. Consequently, in the microbial cell, high levels of iron may lead to the accumulation of iron-storage proteins which harbour iron in a non-reactive insoluble inorganic form (Andrews, 1998; Touati, 2000). In bacteria, there are two types of iron-storage proteins: typical non-haem ferritins with strong similarity to the mammalian ferritin H-chain, and haem-containing bacterioferritins. Though ferritins and bacterioferritins are evolutionarily distinct proteins, they are both composed of 24 subunits (15–22 kDa) that are assembled similarly in a three-dimensional spherical globular shell (Andrews, 1998).

Ferritin has the capacity to accommodate a large amount of iron, about 3000–4000 iron atoms per molecule, which is important for its role in linking cellular iron metabolism and oxidative stress homeostasis (Touati, 2000; Arosio & Levi, 2002; Andrews et al., 2003; Theil, 2003). In bacteria, this link is well studied in Escherichia coli FtnA. By scavenging intracellular free iron, FtnA stores about 50% of total cellular iron upon entry into stationary phase and it protects against Fe(II)-mediated formation of hydroxyl radicals via Fenton chemistry (Abdul-Tehrani et al., 1999; Touati, 2000). This is accomplished through the ferrooxidase centre, which is essential for iron incorporation (Arosio & Levi, 2002; Andrews, 1998). Iron stored in FtnA may become available for metabolism when E. coli is shifted from iron-rich to iron-limiting conditions (Abdul-Tehrani et al., 1999). In this regard, iron-storage proteins are generally synthesized in response to iron-replete conditions in order to prevent excess accumulation of intracellular iron and simultaneously there is a down-regulation of the high-affinity iron uptake mechanism. The ferric uptake regulator, Fur, which uses Fe(II) as corepressor, is the primary regulator controlling iron metabolism (Ratledge & Dover, 2000; Andrews et al., 2003).

In anaerobic bacteria little is known about metal/oxidative stress interactions and little consideration has been given to...
the fact that iron in the anaerobic environment is presumed to be present in the reduced form as Fe(II). This is probably because free Fe(II) can predominate over Fe(III) in the absence of oxygen, and being soluble even at neutral pH it is likely to be readily available. Furthermore, the absence of oxygen lowers the risk of toxic radical generation. Nevertheless, this may be an oversimplification since a ferritin-like homologue which harbours iron under anaerobic conditions is present in "Porphyromonas gingivalis" (Rocha et al., 1992). Recently, a ferritin from the closely related periodontal pathogen "Porphyromonas gingivalis" and a bacte-rioferritin from the sulfate-reducing bacterium "Desulfovibrio desulfuricans" have been cloned and characterized (Ratnayake et al., 2000; Romao et al., 2000). This provides evidence that mobilization and storage of non-haem iron by anaerobic bacteria may be more complex than previously thought. It has been assumed that in anaerobic organisms ferritins are an important component of an oxygen defence mechanism that protects against the deleterious effect of uncontrolled ferrous iron oxidation (Rocha et al., 1992; Romao et al., 2000). Moreover, the presence of both oxygen-detoxifying enzymes and iron-storage mechanisms in anaerobes is essential for their survival in an adverse oxygen-rich environment (Romao et al., 2000; da Costa et al., 2001).

When shifted from anaerobic to aerobic conditions, the growth of "Bacteroides" spp. is immediately halted and the bacteria enter a stationary-phase-like mode (Rocha et al., 1996, 2003; Pan & Imlay, 2001). Simultaneously, an extensive response is immediately induced, and a significant shift in protein expression patterns has been shown to be important for protection and survival under aerobic conditions (Rocha et al., 1996). Control of this complex oxidative stress response is not yet fully understood but an important component of it, the peroxide-inducible response, has been shown to be regulated by the redox transcriptional regulator OxyR (Rocha et al., 2000). OxyR-independent oxygen-dependent control of the response also has been demonstrated, indicating that there are additional levels of control of the oxidative stress response (Rocha et al., 2003). In aerobic and facultative bacteria, it has been shown that the control of iron metabolism and the oxidative stress response are linked via a number of transcriptional regulators (Chen et al., 1995; Bereswill et al., 2000; Touati, 2000; Massé & Gottesman, 2002; Fong et al., 2003) but the influence and role of iron in the "B. fragilis" oxidative-stress response has not been given much attention. Iron transport and mobilization in the cell are absolutely key to controlling the levels of free iron and subsequent damage from oxygen radicals. It is known that "B. fragilis" responds to iron limitation with an iron-induced expression of several proteins on the cell surface (Otto et al., 1988) and it produces an iron-storage protein, ferritin, that can incorporate radiolabelled iron in vivo (Rocha et al., 1992). Thus it appears that "B. fragilis" has a well-developed iron-responsive regulatory network, but this system has not yet been elucidated. In this study we present data showing that "B. fragilis" ferritin, FtnA, is up-regulated at the transcriptional level in the presence of excess iron in an oxidative environment but not in reduced anaerobic conditions, and that the peroxide regulator OxyR is involved in "B. fragilis" ferritin regulation.

**METHODS**

**Strains and growth conditions.** "B. fragilis" strains used in this study are shown in Table 1. Strains were routinely grown anaerobi-cally in brain heart infusion broth supplemented with haemin, cysteine and NaHCO3 (BHIS). Rifampicin (20 µg ml\(^{-1}\)), gentamicin (100 µg ml\(^{-1}\)), tetracycline (5 µg ml\(^{-1}\)) and erythromycin (10 µg ml\(^{-1}\)) were added to the medium when required. For viable-cell count assays, bacteria were grown to mid-exponential phase, diluted in BHIS broth, plated on medium containing gentamicin, and incubated anaerobically for 3–5 days. For some experiments, a semi-defined medium (SDM) was used by adding 0.5 g tryptone l\(^{-1}\) to a modification of the chemically defined medium of Varel & Bryant (1974). The modified medium composition was as follows: KH\(_2\)PO\(_4\), 1.15 g l\(^{-1}\); (NH\(_4\))\(_2\)SO\(_4\), 0.4 g l\(^{-1}\); NaCl, 0.9 g l\(^{-1}\); L-methionine, 75 mg l\(^{-1}\); MgCl\(_2\).6H\(_2\)O, 20 mg l\(^{-1}\); CaCl\(_2\).2H\(_2\)O, 6.6 mg l\(^{-1}\); MnCl\(_2\).4H\(_2\)O, 1 mg l\(^{-1}\); CoCl\(_2\).6H\(_2\)O, 1 mg l\(^{-1}\); resazurin, 1 mg l\(^{-1}\); L-cysteine, 50 mg l\(^{-1}\); haemin, 5 mg l\(^{-1}\), glucose, 5 g l\(^{-1}\). Twenty millilitres of 10% NaHCO\(_3\) were added per litre of medium, final pH 7.2. Haemin was replaced by 5 µg protoporphyrin IX ml\(^{-1}\) when required.

To induce peroxide stress, cultures were grown to an OD\(_{550}\) of 0.3 and treated with appropriate concentrations of H\(_2\)O\(_2\) for 5 min prior to total RNA extraction. To induce oxygen stress, cultures were split in half; one half was kept anaerobically and the other half was shaken aerobically at 250 r.p.m. in a volume/flask ratio of 1:5. Aliquots were taken at appropriate time points for total RNA extraction. Addition of 50 µM 2,2'-bipyridyl and 100 µM desferrioxamine was used to restrict iron availability in SDM. For some redox-stress experiments, BHI without the addition of cysteine and resazurin was used instead of chemically defined medium, which requires the presence of the reductant cysteine to support growth (Varel & Bryant, 1974). Ferrous sulfate, potassium ferricyanide and diamide were added when required as indicated in the text.

**Cloning, DNA sequencing and construction of ftnA insertion mutant.** All DNA modifications and manipulations were carried out according to standard protocols (Sambrook et al., 1989). To amplify the "B. fragilis" ftnA homologue the following oligonucleotide primers were designed based on conserved amino acid sequence residues of bacterial ferritins available from GenBank. The sense and antisense oligonucleotide sequences are as follows: 5'GAR CAR ATH WSI GCI GAR ATG TGG-3' and 5'-YTC YTC YTC IWS YTG YTC-3'. A 375 bp fragment was then amplified from the chromo-some by Taq polymerase using a PCR amplification kit. The amplified fragment was extracted from an agarose gel and ligated into the HinClI site of the suicide vector pFD516 (Smith et al., 1995), resulting in pFD775. The new construct, pFD775, was mobilized from E. coli DH10B into "B. fragilis" ftnA389 (Privitera et al., 1979) by aerobic triparental filter mating protocols (Shoemaker et al., 1986). The transconjugants were selected on BHIS agar plates containing 20 µg rifampicin ml\(^{-1}\), 100 µg gentamicin ml\(^{-1}\) and 10 µg erythromycin ml\(^{-1}\). The "B. fragilis" ftnA::pFD516 insertion mutant, strain IB335, was subjected to Southern blot hybridization analysis to confirm the single cross-over disruption of the target gene. A dps oxyR double mutant strain was constructed by mobilizing pFD759 from E. coli DH10B into "B. fragilis" ftnA389 by triparental mating protocol as described above. pFD775 was inserted into the oxyR and dps oxyR strains by triparental mating to create oxyR ftnA and dps oxyR ftnA mutants respectively. The strategy to isolate the entire ftnA gene region was to rescue the suicide vector pFD775 from the
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Phenotype*</th>
<th>Reference</th>
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<tr>
<td><strong>B. fragilis</strong></td>
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<tr>
<td>638R</td>
<td>Clinical isolate, Rif</td>
<td>Privitera et al. (1979)</td>
</tr>
<tr>
<td>IB298</td>
<td>638R ΔoxyR:: tetQ, Rif Tet</td>
<td>Rocha et al. (2000)</td>
</tr>
<tr>
<td>IB305</td>
<td>638R ΔoxyR, Δdps::tetQ, Rif Tet</td>
<td>This study</td>
</tr>
<tr>
<td>IB335</td>
<td>638R ftnA::pFD516, Rif Erm</td>
<td>This study</td>
</tr>
<tr>
<td>IB336</td>
<td>638R Δdps::tetQ, Rif Tet</td>
<td>This study</td>
</tr>
<tr>
<td>IB345</td>
<td>638R ΔoxyR, Δdps::tetQ ftnA::pFD516, Rif Tet Erm</td>
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</tr>
<tr>
<td>IB346</td>
<td>636R ftnA + ftnA::xyIB</td>
<td>This study</td>
</tr>
<tr>
<td>IB348</td>
<td>IB298 ftnA + ftnA::xyIB</td>
<td>This study</td>
</tr>
<tr>
<td>IB388</td>
<td>IB298 ftnA::pFD516, Rif Tet Erm</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFD516</td>
<td>Suicide vector, derived from deletion of pBl143 in pFD288, (TetX) (Sp) Erm</td>
<td>Smith et al. (1995)</td>
</tr>
<tr>
<td>pFD700</td>
<td>600 bp TaqI internal fragment of bglA cloned into the Clal site of pFD516, (Sp) Erm</td>
<td>Rocha et al. (2000)</td>
</tr>
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<td>pFD750</td>
<td>2.7 kb DNA fragment containing the dps oxyR chromosomal region from strain 638R cloned into the BamHI/EcoRI sites of pFD516</td>
<td>Rocha et al. (2000)</td>
</tr>
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<td>pFD759</td>
<td>587 bp SalI/MscI fragment from pFD750 deleted and replaced with a 2.4 kb tetracycline-resistance gene, tetQ, to create a dps oxyR double mutant</td>
<td>This study</td>
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<td>pFD775</td>
<td>375 bp ftnA internal fragment cloned into the unique HinIII site of pFD516, (TetX) (Sp) Erm</td>
<td>This study</td>
</tr>
<tr>
<td>pFD812</td>
<td>Reporter gene vector, 1.2 kb EcoRI fragment with promoterless β-xylosidase gene (xyIB) cloned into EcoRI site of pFD700 with a 300 bp ftnA promoter region into the BamHI/EcoRI sites, (Sp) Erm</td>
<td>This study</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
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<tr>
<td>DH10B</td>
<td>F' mcrA Δ(mrr-hsdRMS-mcrBC) &amp;80lacZAM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galK rpsL(StrΔ3) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
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</table>

*Erm, erythromycin resistance; Cfx, cefoxitine resistance; Rif, rifampicin resistance; Tet, tetracycline resistance; Sp, spectinomycin resistance. Parentheses indicate antibiotic resistance expression in *E. coli.*

RNA extraction, Northern blot hybridization and primer extension. Total RNA extraction and Northern blot analysis of mRNA were carried out as previously described (Rocha & Smith, 1997) and an internal fragment of ftnA was used as specific probe. Densitometry analysis of the autoradiograph was normalized to the relative intensity of total 23S and 16S RNA detected on the ethidium-bromide-stained agarose gel to correct for any loading differences.

Prime extension analysis was performed on total RNA obtained from mid-exponential-phase cells of *B. fragilis* 638R grown anaerobically and then subjected to oxidative stress conditions as described previously (Rocha & Smith, 1997). ftnA-specific oligonucleotides, 5'-GGCTTTCCATCCAGTGTGC-3' complementary to nucleotides 117–135 and 5'-GGGATAAGCATGCCCATCTCTTGCC-3' complementary to nucleotides 143 to 167 of the ftnA coding region, were used to map the 5'-end of the extended product. The oligonucleotides were labelled with [γ-³²P]ATP and used as primers for the reverse transcriptase reaction as described previously (Rocha & Smith, 1997). The extended labelled products were electrophoresed on 8% polyacrylamide gels containing urea. A nucleotide sequence ladder was prepared with Sequenase 2.0 (USB) using a template covering the transcription start site region, with the same oligonucleotide that was used for the reverse transcription reactions.

Construction of *ftnA*: *xyIB* transcriptional fusions. A 300 bp fragment encompassing 249 bp upstream and 51 bp downstream of the ftnA translational start codon was PCR amplified using High-fidelity *Taq* DNA polymerase with the oligonucleotides 5'-GGGTG-GGGATCCGATAGCC-3' and 5'-CTCGGTAAATCTGTCG-3', containing restriction sites for *BamHI* and EcoRI (in italic) respectively. The 300 bp *BamHI*/EcoRI fragment was cloned into the *BamHI*/EcoRI sites of pFD700 (Rocha et al., 2000) and the 1.2 kb EcoRI fragment from a promoterless β-xylosidase/s-arabinosidase bifunctional reporter gene (Whitehead, 1997) was cloned into the unique EcoRI site of the new construct pFD812. Plasmid pFD812 was mobilized from *E. coli* DH10B into *B. fragilis* strains by aerobic triparental filter mating as mentioned above and integrated into the *B. fragilis* chromosome bglA locus (Rocha et al., 2000).

Enzyme assays. The β-xylosidase assays in crude extracts were performed using *p*-nitrophenyl β-D-xylopyranoside as substrate as previously described (Rocha et al., 2000). One unit of β-xylosidase is the amount of enzyme which releases 1 μmol *p*-nitrophenol min⁻¹ at 37 °C.
RESULTS

Analysis of the ftnA nucleotide sequence

In a previous study, a B. fragilis ferritin-like protein was isolated and characterized (Rocha et al., 1992). In this study, the ferritin gene was cloned from the B. fragilis 638R chromosome by PCR and analysis of the nucleotide sequence revealed an ORF of 480 bp (GenBank accession no. AY023871). This ORF encodes a protein of 159 amino acids with a predicted molecular mass of 18 064. Comparison of the N-terminal amino acid sequence of the purified ferritin (Rocha et al., 1992) to the ftnA ORF revealed a perfect match for the first 30 residues. Thus, the identities of the gene and gene product were confirmed. When FtnA was compared to other ferritins, it showed the highest amino acid identity to P. gingivalis Ftn (55 % identity, 67 % similarity) (Fig. 1). In addition, the alignment in Fig. 1 indicates that the ferroxidase centre residues of bacterial ferritins (Andrews, 1998) are conserved in B. fragilis ferritin.

The phylogenetic relationship between B. fragilis ftnA and 18 ferritins from animals, plants, bacteria and archaea was determined from a progressive multiple alignment of the amino acid sequences followed by parsimony analysis. This comparison showed that B. fragilis FtnA is grouped with P. gingivalis Ftn in a cluster with members of the Archaea, in agreement with the previous report of P. gingivalis ferritin (Ratnayake et al., 2000) (data not shown).

ftnA regulation under anaerobic and aerobic conditions

To investigate the effect of iron availability on the expression of ferritin, Northern hybridization analysis was performed with RNA extracted from mid-exponential-phase cultures grown under iron-replete and iron-limiting conditions. Anaerobic cultures grown under the iron-limiting conditions showed a lower growth rate than the iron-replete cultures (Fig. 2b). ftnA was transcribed as a monocistronic message of approximately 600 nt. Under anaerobic conditions, the basal level of ftnA mRNA was not dependent on the ferrous iron content in the growth medium (Fig. 2a). In

Fig. 1. Multiple alignment of the B. fragilis deduced amino acid sequence for ferritin with other bacterial, archaelal and mammalian (H-chain) ferritin homologues. Archeoglobus fulgidus (B69354), Campylobacter jejuni (S75778), E. coli (P23887), Haemophilus influenzae (P43708), Helicobacter pylori (CAA76033), Methanobacterium thermoautotrophicum (G69077), Popyphromonas gingivalis (BAA36191), Thermotoga maritima (E72293), human ferritin heavy chain (P02794). Conserved amino acid residues (>50% identity) are labelled with black boxes. Semi-conserved amino acid substitutions are depicted by grey boxes. Key ferroxidase centre residues of the E. coli FtnA (Glu-17, Tyr-24, Gln-32, Glu-94; Gln-127 and Glu-130) and mammalian H-chain ferritin (Glu-27, Tyr-34, Glu-61, Glu-62, His-65, Glu-107 and Gln-141) (Andrews, 1998) are depicted by the amino acid one-letter code and an asterisk above the sequence respectively. Alignment of peptide sequences was performed using the GCG programs PILEUP and BOX with the peptide scoring matrix default data file blosum62.cmp for the comparison of amino acids substitution.
contrast, ftnA mRNA was induced nearly 10-fold following oxygen exposure in iron-replete conditions compared to anaerobic culture controls. However, in iron-limiting cultures, the induction of ftnA mRNA by oxygen was lower (about fourfold) compared to anaerobic culture controls. This suggests that the oxidative stress induction of ftnA mRNA induction following a shift of the culture from anaerobic to aerobic conditions is dependent on the effect of oxygen while P2 is up-regulated by a combined effect of iron and oxygen.

**Effect of shift in the redox conditions**

To investigate whether redox conditions affect regulation of the ftnA P2 promoter, experiments were carried out with cultures grown in rich medium without addition of cysteine and treated with the oxidants potassium ferricyanide, hydrogen peroxide and diamide. When cultures were treated with H$_2$O$_2$ or oxygen there was a threefold and eightfold increase respectively in P2 expression compared to anaerobic cultures, suggesting that oxidative stress also affects ftnA expression in medium without the reductant cysteine (Fig. 3b). Strong evidence confirming that a shift in the redox conditions affects ftnA mRNA levels was obtained when potassium ferricyanide was added to mid-exponential-phase cultures. The P2 promoter was induced about fourfold and 5-5-fold following addition of 100 mM FeSO$_4$ and 1 mM ferricyanide, similar to the response obtained when untreated anaerobic cultures were shifted to aerobic conditions (Fig. 3b). In contrast, addition of iron in the ferrous form did not alter the ratio of P2 expression relative to the levels of P1 seen in the anaerobic untreated controls (Fig. 3b).

Free thiol groups play an important metabolic role in anaerobic bacteria, and anaerobic oxidation of thiols by diamide mimics the effect of oxygen on these organisms (Morris, 1975). Thus, to investigate the participation of thiol oxidation in the regulation of ftnA mRNA expression, cultures were treated with diamide, and the ftnA mRNA levels were analysed by Northern hybridization. In the absence of oxygen, ftnA expression was induced following diamide treatment in a dose-dependent manner up to 500 μM (Fig. 4). There was about twofold induction when cultures were treated with 50 μM H$_2$O$_2$ for 5 min or exposed to oxygen for 1 h prior to total RNA extraction are indicated by the arrows.
with 500 μM diamide compared to anaerobic untreated control culture.

**OxyR and ftnA mRNA expression**

The results above suggest that an imbalance in the redox thiol/disulphide couples in *B. fragilis* does indeed mimic the effect of oxygen in *ftnA* expression. This prompted us to investigate whether the redox-sensing transcriptional regulator, OxyR, would affect *ftnA* expression. The effect of an *oxyR* deletion on *ftnA* expression was measured by analysis of an *ftnA*::*xylB* transcriptional fusion. The induction of *ftnA* by oxygen, measured as β-xylosidase activity, was reduced from 50 U (mg protein)⁻¹ in the parent strain to about 16 U (mg protein)⁻¹ in an *oxyR* mutant (Fig. 5). Nevertheless, *ftnA* [anaerobic level 7 U (mg protein)⁻¹] was still induced following oxygen exposure [to 16 U (mg protein)⁻¹] in the ΔoxyR strain, suggesting that *ftnA* may be under dual regulation by OxyR and OxyR-independent mechanisms. Further evidence for this was obtained when cultures were treated with diamide (Fig. 6). *ftnA* expression was induced about 2.5-fold in the parent strain following treatment with 500 μM diamide compared to the level observed in the untreated culture. This moderate induction,
however, was less pronounced in the oxyR mutant strain, as was the basal level in the untreated culture.

**Effect of ftnA on oxygen tolerance**

There was no effect of oxygen exposure on cell viability in an ftnA mutant compared to the parent strain (Fig. 7). In addition, single deletion mutants in oxyR or the non-specific DNA binding protein gene, dps, and the ftnA oxyR double mutant strain showed no differences in oxygen tolerance compared to the parent strain (Fig. 7b). In contrast, an oxyR dps double mutant showed about a 1 log decrease in cell viability within 48 h of oxygen exposure and no viable cells were present after 98 h. However, the decrease in cell viability was more pronounced in an ftnA dps oxyR triple mutant, where an approximately 3 log decrease in cell viability was seen at 48 h and no survival at 72 h following oxygen exposure. Moreover, none of the strains tested showed any loss of viability compared to the parent strain.

**Fig. 4.** Autoradiograph of Northern hybridization of total RNA from mid-exponential-phase *B. fragilis* 638R grown in BHIS without addition of cysteine. Diamide was added at the concentrations indicated above the panel. The probe was an ftnA internal gene fragment. The approximate size of the transcripts is indicated.

**Fig. 5.** Expression of ftnA induced by oxidative stress. β-Xylosidase activity was determined in the crude extracts from mid-exponential-phase cultures of *B. fragilis* wild-type (WT) strain 638R ftnA::xylB and in the oxyR mutant strain 638R ΔoxyR ftnA::xylB. Bacteria were exposed to different oxidative stress conditions following the addition of the thiol oxidizing agent diamide. Dark grey bars, anaerobic culture control; light grey bars, culture treated with 100 μM diamide for 30 min; white bars, culture treated with 500 μM diamide for 30 min. The data presented are the mean of five independent experiments. Error bars represent the standard deviation of the mean.

**Fig. 6.** Expression of ftnA induced by diamide. β-Xylosidase activity was determined in the crude extracts from mid-exponential-phase cultures of *B. fragilis* wild-type (WT) strain 638R ftnA::xylB and in the oxyR mutant strain 638R ΔoxyR ftnA::xylB. Bacteria were exposed to disulfide stress conditions following the addition of the thiol oxidizing agent diamide. Dark grey bars, anaerobic culture control; light grey bars, culture treated with 100 μM diamide for 30 min; white bars, culture treated with 500 μM diamide for 30 min. The data presented are the mean of five independent experiments. Error bars represent the standard deviation of the mean.

**Fig. 7.** Survival of mid-exponential-phase anaerobic cells of *B. fragilis* strains shifted to aerobic conditions. Cultures of mid-exponential-phase cells at an OD_{500} of 0·3 were divided at time zero; one half was maintained anaerobically (a) and the other half was shaken at 250 r.p.m. in air at 37 °C (b). Viable cell counts were determined at times shown. ■, Wild-type; ●, oxyR; ○, dps; +, ftnA; ▲, oxyR dps; Δ, oxyR ftnA; □, oxyR dps ftnA. The data presented are representative of the results from two independent experiments.
when control cultures were maintained in anaerobic conditions (Fig. 7a).

**DISCUSSION**

Anaerobic bacteria do not grow in the presence of atmospheric oxygen, although some can grow at very low oxygen concentrations (Loesche, 1969; Baughn & Malamy, 2004). Thus when shifted from anaerobic to aerobic conditions B. fragilis must ‘redirect’ its energy resources and metabolism to protection against oxidative stress. Consistent with this there is a significant shift in the protein expression pattern that has been shown to be important for survival under aerobic conditions (Rocha et al., 1996). Though most of these stress proteins are of unknown function, a better understanding has started to emerge. The peroxide response genes katB, dps, ahpCF, tpX, rbr-2 and rpbA are part of the OxyR-regulated peroxide-response regulon (Rocha et al., 2000, 2003; Herren et al., 2003) and in this study we show that ftmA also is a component of the OxyR regulatory system. Comparison of the FtnA deduced amino acid sequence and the presence of conserved ferroxidase residues confirm that this is a bacterial ferritin and also confirm a previous study reporting the presence of a ferritin-like protein in this anaerobe (Rocha et al., 1992). B. fragilis ftmA is regulated at the transcriptional level and is constitutively expressed under anaerobic conditions regardless of the iron content in the medium. In contrast, ftmA was up-regulated in excess iron upon oxygen exposure, suggesting that iron and oxygen induce ftmA expression in concert. In addition, a similar effect was observed following oxidation of free thiol by diamide in the absence of oxygen, suggesting that a shift in the redox conditions does indeed affect ftmA mRNA synthesis. Although the results of the present study indicate that induction of ftmA is regulated at the transcriptional level, a mechanism involving increased stability of mRNA cannot be conclusively ruled out at this point.

In facultative and aerobic bacteria control of iron metabolism and the oxidative stress response are linked via several transcriptional and post-transcriptional regulators (Chen et al., 1995; Bereswill et al., 2000; Horsburgh et al., 2001; Hirosue et al., 2001; Massé & Gottesman, 2002). In *E. coli*, a Fur-regulated small RNA, RyhB, controls the expression of FtnA and Bfr (Massé & Gottesman, 2002) while in *Helicobacter pylori*, Fur activates the expression of Pfr (ferritin) by binding directly to the pfr promoter sequence (Bereswill et al., 2000; Delany et al., 2001). Recently, the dual ferritins genes, ftmAB, of *Actinobacillus actinomycetemcomitans* were shown to be induced by oxygen with complex transcriptional regulation controlled by the aerobic/anaerobic sensor ArcB, the quorum-sensing autoinducer LuxS (Fong et al., 2003) and possibly FNR, an oxygen-sensing transcriptional regulator of aerobic and facultative bacteria (Hirosue et al., 2001). The importance of a co-integrated regulation of iron homeostasis and oxidative stress has been demonstrated in vivo for *Staphylococcus aureus*. The Fur-like repressor of the peroxide response, Per, regulates the iron-storage protein ferritin in addition to several other oxidative stress proteins (Horsburgh et al., 2001). In this report we showed that the B. fragilis oxidative stress response is also linked to iron metabolism through the thioldisulfide redox regulator of the peroxide response, OxyR. However, we believe that the OxyR-mediated control is in fact indirect, since analysis of the ftmA promoter region did not show an OxyR nucleotide-binding motif sequence present in the promoter regions of B. fragilis peroxide-response genes ahpCF, katB, dps, tpX, rbr-2 and rpbA (data not shown). Perhaps the effect of OxyR on ftmA expression occurs via its interaction with other regulatory mechanisms involved in iron homeostasis, as has been demonstrated to occur in *E. coli* (Zheng et al., 1999).

Although the *E. coli* ftmA mutant has no apparent redox-stress sensitivity, a strong indication that disturbing iron homeostasis leads to increased sensitivity to oxidative stress was demonstrated in an ftmA fur double mutant, which is more sensitive to hydroperoxides than are the single mutant strains (Abdul-Tehrani et al., 1999). In addition, overexpression of FtnA compensates for the deleterious effect of fur deletion (Touati, 2000). In *B. fragilis* we also found that a mutation in the ftmA gene alone showed no loss of viability upon oxygen exposure, in agreement with data reported for ftin and dps ftin double mutants in the closely related organism *P. gingivalis* (Ushima et al., 2003). However, a role for iron detoxification during aerotolerance in *B. fragilis* was demonstrated when an ftmA mutation in conjunction with a disruption in the dps and oxyR genes amplified the sensitivity of this anaerobe to oxygen exposure (Fig. 7). Additional evidence that ferritin or bacterioferritin participate in protection against iron-mediated oxygen radical cellular damage was obtained in other bacteria such as *H. pylori*, *Campylobacter jejuni*, and *Neisseria gonorrhoeae*, where disruption of the iron-storage mechanism increased bacterial sensitivity to oxidative stress *in vitro* (Wai et al., 1996; Chen & Morse, 1999; Waidner et al., 2002) and the ability to colonize tissues *in vivo* (Waidner et al., 2002).

Ferritin isolated from *B. fragilis* grown under anaerobic conditions contains about three iron atoms per molecule (Rocha et al., 1992). The bacterioferritin from the strict anaerobe *D. desulfuricans* contains two iron atoms per monomer (Romao et al., 2000). This low iron content does not correlate with their role in iron-storage but it is presumed that they may play a role in detoxification during transient exposure to oxygen (Rocha et al., 1992). This may be related to the fact that in anaerobic conditions, ferrous iron is readily available and toxicity of excess iron is likely not a problem. On the other hand, in the presence of oxygen, free ferrous iron will be unavoidably converted to its ferric form, with potential toxicity through generation of oxygen-derived cell-damaging free radicals. Thus induction of ferritin synthesis upon oxygen exposure correlates with the need for *B. fragilis* to maintain excess iron in a non-toxic form.
In conclusion, this study presents evidence that regulation of *B. fragilis* finA expression occurs at the transcriptional level. In addition, it was demonstrated that iron, oxygen and thiol oxidation are involved in the modulation of *B. fragilis* finA expression; however, the molecular mechanisms involved in the transcriptional regulation are not yet known and will be the subject of further investigation. This study confirms and adds to previous reports indicating that the oxidative stress response in this aerotolerant anaerobe involves a complex genetically regulated response.

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**REFERENCES**


