Thioredoxin Reductase Is Essential for Thiol/Disulfide Redox Control and Oxidative Stress Survival of the Anaerobe Bacteroides fragilis

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Received 6 May 2007/Accepted 3 September 2007

Results of this study showed that the anaerobic, opportunistic pathogen Bacteroides fragilis lacks the glutathione/glutaredoxin redox system and possesses an extensive number of putative thioredoxin (Trx) orthologs. Analysis of the genome sequence revealed six Trx orthologs and an absence of genes required for synthesis of glutathione and glutaredoxins. In addition, it was shown that the thioredoxin reductase (TrxB)/Trx system is the major or sole redox system for thiol/disulfide cellular homeostasis in this anaerobic bacterium. Expression of the B. fragilis trxB gene was induced following treatment with diamide or H2O2 or exposure to oxygen. This inducible trxB expression was OxyR independent. Northern blot hybridization analysis showed that the trxB mRNA was cotranscribed with lolA as a bicistronic transcript or was present as a monocistronic transcript that was also highly induced under the same conditions. The role of LolA, a prokaryotic periplasmic lipoprotein-specific molecular chaperone in the thiol/disulfide redox system, is unknown. A trxB deletion mutant was more sensitive to the effects of diamide and oxygen than the parent strain. In addition, the trxB mutant was unable to grow in culture media without addition of a reductant. Furthermore, the trxB mutant was not able to induce intraabdominal abscess formation in a mouse model, whereas the parent strain was. Taken together, these data strongly suggest that TrxB/Trx is the major, if not the sole, thiol/disulfide redox system in this anaerobe required for survival and abscess formation in a peritoneal cavity infection model.

Adaptation of anaerobic bacteria to aerobic environments has taken different courses in different families of anaerobes. Some bacteria did not adapt and remained sensitive to aerobic environments. The bacteria that adapted had to cope with the problem of thiol oxidation and developed different mechanisms to deal with the presence of oxygen (10). One of the latter bacteria is Bacteroides fragilis, a human intestinal obligate anaerobe which is the anaerobe most frequently isolated from human infections. B. fragilis possesses a complex oxidative stress response mechanism which is required to maintain extended aerotolerance. A set of at least 28 proteins is synthesized in response to treatment with hydrogen peroxide or oxygen exposure. Some of these proteins, such as KatB, Dps, and AhpCF, have been identified (16, 37, 39, 40), but the mechanism that confers aerotolerance in this anaerobic bacterium still remains to be elucidated. In the presence of low, nanomolar concentrations of oxygen, B. fragilis can maintain nearly normal growth and metabolic activity by consuming oxygen (3). This activity is not sufficient to allow growth under fully aerobic conditions, in which a wide range of cellular functions are inhibited (19). One of the cellular functions sensitive to oxygen is the thiol/disulfide redox balance. Thiols are oxidized within minutes following exposure to air, and the oxidation of thiols produces reduced oxygen species that can be highly toxic (10). Iron-sulfur proteins also are very reactive, and it has been shown that the iron-sulfur clusters present in many metabolic enzymes of Bacteroides spp. are vulnerable to oxygen exposure and that this affects their aerotolerance response (19, 32).

The intracellular environment is characterized by reducing conditions generally due to the presence of small proteins with redox-active cysteine residues, including the thioredoxins (Trxs), the glutaredoxins (Grxs), the monocysteine tripeptide glutathione (GSH), and other low-molecular-weight thiols (10, 17). Trxs and Grxs are small ubiquitous thiol/disulfide oxidoreductases containing the two-cysteine redox motif CXXC. Trx contains this motif as WCGPC, while most Grxs contain the CPYC motif (31, 55). Trxs and Grxs have been implicated in a variety of physiological processes and biological pathways. In addition, they play a role in defense against oxidative stress, either by reducing protein disulfide bonds produced by various oxidants or by scavenging reactive oxygen species. Trxs and Trx reductases (TrxB) are widespread in all phylogenetic branches, including viruses, archaea, bacteria, protozoans, yeasts, plants, and animals (2), while GSH is absent in many bacteria, including some facultative gram-positive and anaerobic bacteria (9, 30, 47, 52).

The reduction of intracellular disulfides is mediated by NADPH, which operates together with flavin adenine dinucleotide-dependent TrxB to convert oxidized Trx to the free thiol form (2). This cellular redox mechanism is likely to be essential for adaptation of aerotolerant anaerobes to aerobic environments since they require mechanisms to minimize the occurrence of thiol oxidation and to mitigate its consequences. TrxB/Trx systems have been identified in some anaerobe species, including Desulfovibrio desulfuricans (45), Clostridium pasteur-
Plasmids

**E. coli** DH10B

Cloning host strain

Invitrogen

<table>
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<th>Strain or plasmid</th>
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<td><strong>B. fragilis</strong> strains</td>
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<td>pFD516</td>
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<td>pFD754</td>
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<td>pFD846</td>
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<td>This study</td>
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<td>pFD874</td>
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<td>This study</td>
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<td>pFD892</td>
<td>1.285-bp promoterless trxB fragment amplified from the 638R chromosome was cloned into expression vector pFD340</td>
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<sup>a</sup> Erm<sup>b</sup>, erythromycin resistance; Rif<sup>a</sup>, rifamycin resistance; Sp<sup>a</sup>, spectinomycin resistance; Amp<sup>a</sup>, ampicillin resistance. Parentheses indicate antibiotic resistance expression in **E. coli**.

**Materials and Methods**

**Bacterial strains and growth conditions.** *B. fragilis* strains used in this study are listed in Table 1. Strains were grown anaerobically in brain heart infusion (BHI) broth supplemented with hemin, cysteine, and NaHCO₃ (BHIS) for routine cultures. Rifamycin (20 μg/ml), 100 μg/ml gentamicin, 5 μg/ml tetracycline, 10 μg/ml erythromycin, and 25 μg/ml cefoxitin were added to the media when required. Semidifined medium (SDM) was prepared by adding 0.5 g/liter tryptone to a modified chemically defined medium of Varel and Bryant (53). The modified medium composition was as follows: KH₂PO₄, 1.15 g/liter; NH₄SO₄, 0.4 g/liter; NaCl, 0.9 g/liter; t-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; t-cysteine, 500 mg/liter; hemin, 5 mg/liter; and glucose, 5 g/liter. Twenty milliliters of 10% NaHCO₃ was added per liter of medium, and the final pH was 7.2. *B. fragilis* requires a reduced sulfur source to support growth (53), which was tryptone when t-cysteine was omitted.

**Construction of trxB deletion mutant.** Briefly, a 2.1-kb chromosome fragment containing the *trxB* region was amplified by PCR using oligonucleotides containing modifications to create restriction sites for Sphi and BamHI and then cloned into pUC19 to create pFD846. Next, an internal 60-bp BglII/ClaI fragment from the *trxB* gene was deleted and replaced with a 2.1-kb BamHI/NarI cefoxitin (*cfxA*) resistance gene to construct a ΔtrxB::cfxA deletion in a new plasmid, pFD846/ΔtrxB::cfxA. Then a 4.5-kb Sphi/SstI fragment from pFD846/ΔtrxB::cfxA was cloned into the suicide vector pFD516 (50) to create pFD874. This plasmid was mobilized from *Escherichia coli* DH10B into *B. fragilis* 638R by triparental mating, and exconjugants were selected on BHIS containing 20 μg/ml rifamycin, 100 μg/ml gentamicin, and 25 μg/ml cefoxitin. Sensitivity to erythromycin was determined, and Southern blotting was performed to confirm the double-cross-over allele exchange to create a *trxB* mutant designated IB370.

A Δ*trxB* ΔoxyR mutant was constructed by mobilizing suicide vector pFD754 (containing the ΔoxyR::tetQ deletion (39)) into *B. fragilis* IB370 as described above. Exconjugants were selected on BHIS containing 20 μg/ml rifamycin, 100 μg/ml gentamicin, 5 μg/ml tetracycline, and 25 μg/ml cefoxitin. Sensitivity to either tetracycline/cefloxan or erythromycin was used to identify recombinants that were tetracycline and cefoxitin resistant and erythromycin sensitive. Strain IB372 was selected for further study.

A plasmid constitutively expressing *trxB* was constructed by PCR amplification of a 1.285-bp promoterless *trxB* gene containing 21 bp upstream of the ATG start codon and 255 bp downstream of the stop codon. The promoterless *trxB* gene fragment was cloned into the BamHI site of the *Bacteroides-E. coli* shuttle expression vector pFD340 (49) in the same orientation as the IS431 promoter. The new construct, pFD892, was mobilized into *B. fragilis* IB370 as described above. Transconjugants were selected on BHIS containing 20 μg/ml rifamycin, 100 μg/ml gentamicin, and 10 μg/ml erythromycin.

**RNA extraction, Northern blot hybridization, and primer extension.** For oxidative stress experiments, cultures were grown in BHIS to an A₆₅₀ of 0.3 and treated with 50 μM H₂O₂ for 5 min prior to total RNA extraction. To induce oxygen stress, cultures were split in half; one half was incubated anaerobically, and the other half was shaken aerobically at 250 rpm using a volume/flask ratio of 1/5 as previously described (38). For diamide treatment, mid-log-phase cul-
tures were treated with different concentrations of diamide as indicated below. Total RNA extraction and Northern blot analysis of mRNA were carried out as previously described (38). Internal fragments of trxB and lolA were used as specific probes when required. In a densitometry analysis the intensity of the autoradiograph was normalized to the relative intensity of total 23S and 16S rRNA detected on the ethidium bromide-stained agarose gel to correct for any loading differences.

Primary extension analysis was performed with total RNA obtained from mid-log-phase cells of \textit{B. fragilis} 638R grown anaerobically and then subjected to oxidative stress conditions as described previously (39). A lolA-specific oligonucleotide and a trxB-specific oligonucleotide were labeled with [\textit{\alpha}P]-\textit{dATP} and used as primers for the reverse transcriptase reaction.

**Inhibition of growth by oxygen in a soft agar tube culture.** A 0.1 ml aliquot of an overnight culture of \textit{B. fragilis} in BHIS broth without l-cysteine was mixed with 5 ml of BHIS without added L-cysteine and resazurin containing 0.4% agar at 45°C in screw cap tubes (100 by 13 mm) inside an anaerobic chamber. The tubes were capped with sterile cotton stoppers and incubated aerobically at 37°C for 24 h. The area from the top of the agar down to the edge of visible bacterial growth was considered the zone of growth inhibition by oxygen and was measured.

**GSH determination.** The total GSH/xanthine glutathione (GSSG) content in cells was determined with a cycling enzyme assay utilizing glutathione reductase as described by Howe et al. (18) and Fahey et al. (9), with some modifications. Overnight cultures of \textit{B. fragilis} 638R and \textit{E. coli} HB101 (control strain) were grown in SDM under anaerobic conditions and used to inoculate fresh SDM cultures. Bacteria were grown to mid-log phase (optical density at 550 nm, 0.45), and the cultures were split. One half of each culture was incubated aerobically in a shaker incubator at 37°C for 1 and 24 h. The other half was incubated under anaerobic conditions for the same time periods. The amount of total GSH was determined in mid-log- and stationary-phase cells (24 h of incubation). Cells were washed in phosphate-buffered saline (50 mM phosphate buffer [pH 7.4], 150 mM NaCl). Cell pellets were extracted in boiling 80% ethanol, dried at 105°C, weighed in preweighed tubes, and stored at −20°C until they were needed. The B. fragilis \textit{trxB}, six Trx orthologs, and more than 14 other proteins that are dependent on the redox-active center CXXC motif common to Trxs (8, 17), and four of these orthologs, TrxA, TrxC, TrxE, and TrxF, contain the characteristic motif WCGPC (Fig. 1). TrxC also contains an N-terminal conserved prokaryotic membrane lipoprotein precursor signal peptide (15). The TrxD active site contains WCVYC, while TrxG has a WCPHC motif. In addition, another protein with homology to the Trx fold motif family, tentatively designated TrxX, lacks the reactive redox cysteine residues which were replaced by serine and tyrosine (WSPHY). When \textit{B. fragilis} Trxs homologues were compared to other microbial Trxs using a progressive multiple alignment and parsimony analysis, it was found that except for TrxA, all \textit{B. fragilis} Trx homologues were clustered together (data not shown). TrxA, however, grouped in a cluster together with \textit{Flavobacterium}, \textit{Corinbacterium}, and \textit{Clostridium} Trx homologues.

The \textit{B. fragilis} 638R \textit{trxB} open reading frame contains a 948-bp nucleotide sequence. The deduced amino acid sequence is a 315-amino-acid protein homologous to other bacterial Trx reductases. When \textit{B. fragilis} 638R \textit{TrxB} was compared to other TrxBs, it showed the highest homology to \textit{Bacteroides thetaiotaomicron} VPI-5482 \textit{TrxB} (90% identity and 96% similarity) and \textit{P. gingivalis} \textit{TrxB} (84% identity and 80.5% similarity). High homology was also found to \textit{TrxBs} of \textit{E. coli} (52.2% identity and 62.3% similarity), \textit{Haemophilus influenzae} (51.4 identity and 61.5% similarity), and \textit{Mycobacterium tuberculosis} (49% identity and 59.3% similarity). In addition, the pyridine nucleotide disulfide oxidoreductase class II active site is conserved in \textit{B. fragilis} \textit{TrxB} (data not shown). The phylogenetic relationship between \textit{B. fragilis} \textit{TrxB} and other TrxB homologues from animals, plants, bacteria, and yeasts was determined from a progressive multiple alignment of the amino acid sequences, followed by parsimony analysis. This comparison showed that \textit{B. fragilis} \textit{TrxB} groups with other members of the unique \textit{Cytophaga-Flexibacter-Bacteroides} phylum (see Fig. S1 in the supplemental material).

**GSH determination.** During analysis of the \textit{B. fragilis} \textit{TrxB}/Trx system, we noticed that there were no Grxs or any of the genes required for GSH biosynthesis (\gamma-glutamylcysteine synthetase and glutathione synthase) in the genome. This suggested that the \textit{TrxB}/\textit{Trx} system might be the major or sole intracellular thiol/disulfide redox system in this anaerobe. Consistent with this was the finding that no GSH/GSSG was found in crude extracts of \textit{B. fragilis} from mid-log-phase or stationary-phase cultures (limit of detection, 5 pmol in 50 µl of crude extracts). Cultures exposed to aerobic conditions also lacked detectable GSH/GSSG. In contrast, GSH/GSSG was present in the crude extracts of \textit{E. coli} control cultures; 22.2 and 7.05 µmol/g (dry weight) were found in logarithmic- and stationary-phase aerobic cultures, respectively, and in anaerobic cultures the levels were 2.81 and 4.96 µmol/g (dry weight), respectively. These values were comparable to levels previously described for \textit{E. coli} under aerobic and anaerobic conditions (9).

**Effect of free thiol on growth of a \textit{trxB} mutant.** A \textit{trxB} mutant was constructed to determine if another mechanism was present that could maintain reduced cellular thiols and compensate for loss of the \textit{TrxB}/\textit{Trx} system. The availability of thiol compounds in the media greatly affected growth of the \textit{ΔtrxB} mutant in rich BHIS media and SDM. The \textit{ΔtrxB} mutant was unable to maintain growth in the absence of an exogenous free thiol source in SDM, but addition of cysteine or dithiothreitol (DTT) restored the ability of the \textit{trxB} strain to grow.
The growth defect in rich media was less pronounced, probably because of the greater abundance of reduced sulfur sources compared to SDM. Taken together, these findings suggest that in *B. fragilis* the TrxB/Trx system is the major or sole system for mediating the intracellular thiol/disulfide equilibrium; otherwise, the ΔtrxB mutant would not have been so dependent on added thiol for growth.

(Fig. 2A and 2B). The growth defect in rich media was less pronounced, probably because of the greater abundance of reduced sulfur sources compared to SDM. Taken together, these findings suggest that in *B. fragilis* the TrxB/Trx system is the major or sole system for mediating the intracellular thiol/disulfide equilibrium; otherwise, the ΔtrxB mutant would not have been so dependent on added thiol for growth.

**Regulation of trxB expression by oxidative stress and di-amide.** The trxB gene was the second gene of what appeared to be a two-gene operon that also encoded the lipoprotein chaperone, LolA. The expression of trxB following exposure to oxygen, to hydrogen peroxide, and to different concentrations of the thiol-specific oxidant di-amide was examined by Northern hybridization. The autoradiographs revealed that trxB

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**FIG. 1.** Multiple alignment of the deduced amino acid sequences of *B. fragilis* (Bf) Trx orthologs (TrxA through TrxG plus TrxX) with *E. coli* (Ec) TrxA and TrxC amino acid sequences. The conserved Trx fold containing the CXXC redox active site is indicted by thick lines above and below the amino acid sequence region. A consensus of at least 50% identical amino acid residues is indicated by a black background. Conserved amino acid substitutions are indicated by a gray background. The predicted conserved prokaryotic membrane lipoprotein precursor signal peptide (15) present in TrxC is indicated by a thin line under the amino acid sequence. Alignment of peptide sequences was performed using the GCG programs pileup and box with the peptide scoring matrix default data file blosum62.cmp for comparison of amino acid substitutions.

**FIG. 2.** Growth of *B. fragilis* parent strain 638R and the ΔtrxB mutant in BHIS (A) and in SDM (B). ○, parent strain grown in medium containing cysteine; ▲, parent strain grown in medium without cysteine; ■, trxB mutant grown in medium containing cysteine; □, trxB mutant grown in medium without cysteine; ▲, trxB mutant grown in medium without cysteine but with DTT added at the time point indicated. O.D. 550 nm, optical density at 550 nm.
mRNA was expressed as an approximately 0.96-kb monocistronic transcript and as a less abundant 1.8-kb bicistronic transcript containing the upstream gene lolA (Fig. 3A). Expression of trxB monocistronic mRNA was induced approximately fivefold by both hydrogen peroxide and oxygen compared to aerobic controls (Fig. 3A). Deletion of oxyR did not significantly affect the expression of trxB following oxidative stress, but the basal levels of the trxB transcript were slightly higher (twofold) anaerobically in the oxyR background than in the parent strain. trxB also was induced when cultures were treated with diamide in a dose-dependent manner, indicating that trxB was expressed in response to a thiol/disulfide redox imbalance. Maximum induction of trxB mRNA (approximately sevenfold) was observed with 100 μM diamide compared to untreated controls (Fig. 4). In addition, diamide also induced a 1.8-kb lolA trxB bicistronic mRNA, confirming that lolA is cotranscribed with trxB in B. fragilis following thiol redox stress. When Northern blots were probed with an internal lolA gene fragment, the results confirmed that there was up-regulation of the 1.8-kb lolA trxB bicistronic transcript (Fig. 3B) (unpublished data).

Prime extension analysis of lolA revealed two transcription initiation sites, P1 and P2 (Fig. 5). P1 is a constitutive promoter whose transcription starts at an adenine nucleotide 26 bases upstream of the translation start codon. In contrast, P2 was induced upon exposure to oxidative stress and diamide treatment. P2 transcription starts at a thymine nucleotide 8 bases upstream of the translation start site. A lolA transcriptional fusion with the promoterless β-xylanase reporter gene confirmed that the lolA promoter was induced by oxidative stress (data not shown). In contrast, a trxB transcriptional fusion containing the lolA-trxB intergenic region extending 238 bases upstream from the trxB translational start site revealed that there was no transcriptional initiation directly upstream of trxB (data not shown). Because trxB was also detected as a single mRNA in a Northern blot analysis (Fig. 4A), we performed an RNase protection assay to determine whether there might have been posttranscriptional modification of the lolA trxB bicistronic mRNA. The RNase protection assay used to map the lolA trxB intergenic region showed that two 5′ end protected sites of the trxB mRNA mapped at nucleotide positions −36/−35 and −21/−20 with respect to the trxB translational start site (Fig. 3B and data not shown).

**Sensitivity to diamide and peroxide.** Diamide sensitivity mimics thiol oxidation due to oxygen exposure in anaerobic bacteria (29); therefore, sensitivity to diamide was used to determine whether TrxB plays a role in the thiol/disulfide homeostasis. As shown in Fig. 6, the trxB mutant was more sensitive to diamide in disk diffusion assays than the parent strain and an oxyR mutant. When the trxB mutant was complemented with pFD892, the diamide sensitivity was nearly identical to that of the parent strain. Although the oxyR mutation alone had only a small effect on diamide sensitivity, the oxyR trxB double mutant showed greatly increased sensitivity to diamide compared to the single mutants, especially on the plates exposed to oxygen. This suggests that growth inhibition by diamide in both aerobic and anaerobic conditions was likely due to an imbalance in the cellular thiol/disulfide homeostasis and that there might be a link between thiol redox and peroxide stress responses in this anaerobic bacterium. In this regard, the trxB mutant strain was somewhat more sensitive to hydrogen peroxide stress than the wild type, and this sensitivity was additive with oxyR since the oxyR trxB double mutant was inhibited...
more by hydrogen peroxide than either of the single mutants (Fig. 6).

Sensitivity to oxygen exposure in soft agar tubes. The effect of oxygen on the growth of the trxB strain was examined in BHIS soft agar tubes incubated in air without added cysteine. Clearance of bacterial growth from the top of a soft agar tube showed that the trxB mutant was slightly more sensitive to oxygen diffusion into the agar than the oxyR or parent strain (Fig. 7). The parent and oxyR strains had clear zones to 0.4 cm, while the trxB strain was inhibited by oxygen down to 0.9 cm into the agar medium. Interestingly, the oxyR trxB double mutant strain did not even grow into the bottom of the soft agar (depth, 5.5 cm). Addition of cysteine to the medium was not able to overcome the growth inhibition of the double mutant in this assay. The growth of the oxyR trxB double mutant under anaerobic conditions was similar to that of the parent strain (data not shown).

Abscess formation. When B. fragilis leaves the anaerobic intestinal environment to colonize more oxygenated tissues, such as the peritoneal cavity, it encounters a hostile environment (46). It is presumed that protection from oxidative stress is important for survival in the initial stages of an extraintestinal infection (39, 40). Thus, the ability of a trxB strain to form intraabdominal abscesses in a mouse model was investigated. Compared to the parent strain, the trxB mutant was not able to form abscesses at all infection doses (Table 2). In contrast, the parent strain formed abscesses in some animals at every infection dose tested from 10^6 to 10^8 CFU. Challenge with sterile cecal contents alone was used a control, and no abscesses were observed.

DISCUSSION

In this study we demonstrated that the anaerobic opportunistic pathogen B. fragilis lacks the GSH/Grx system and possesses an extensive number of putative Trx orthologs. In addition, we showed that the TrxB/Trx system is apparently the major or sole system for the thiol/disulfide cellular equilibrium in this bacterium. While both the GSH/Grx and TrxB/Trx systems are generally present in facultative gram-negative bacte-
ria, the lack of GSH/Grx in Bacteroides is consistent with reports showing the absence of detectable GSH in other anaerobic bacteria (9). The TrxB/Trx system seems to be the major component for thiol/disulfide balance in anaerobes, as well as in gram-positive organisms such as Bacillus subtilis, Staphylococcus aureus, and Lactobacillus lactis (47, 52, 54). TrxB homologues are essential for growth in both B. subtilis and S. aureus (47, 52) but not in L. lactis, where externally provided GSH or a reducing agent can partially restore growth in a trxB1 mutant (54). Growth of the B. fragilis trxB mutant in SDM could be restored only in the presence of reducing agents, such as L-cysteine or DTT, while the growth deficiency in rich media was less pronounced. This finding could have been due to the presence of free GSH in the media or just the greater overall reducing potential of rich media. Overall, these findings indicate that TrxB is the major component in the reduction of oxidized Trxs in B. fragilis since the presence of an alternative thiol-reducing system was not evidently present to compensate for the lack of a functional TrxB.

The induction of trxB mRNA by atmospheric oxygen and by the specific thiol oxidant diamide indicates that TrxB is an important component of the B. fragilis response to oxidative stress and cellular thiol/disulfide redox imbalance. These findings correlate with the fact that TrxB is required for maximal growth during oxidative stress in vitro (Fig. 6 and 7) and is essential for survival in vivo in an adverse aerobic environment (Table 2). The oxidative stress regulator of trxB expression was not identified in this work, but OxyR did not appear to be directly involved. The OxyR regulon, however, worked together with TrxB to provide maximum protection, as indicated by the additive effect that the trxB oxyR double mutations had.

TABLE 2. Ability of B. fragilis strains to form intraabdominal abscesses in a mouse model

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<th>Strain</th>
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<th>No. of animals with abscesses/total no.</th>
<th>% of animals with abscesses</th>
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<td>Wild type</td>
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<tr>
<td>Control</td>
<td>$1 \times 10^8$</td>
<td>0/8</td>
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a The control received only a 1:10 dilution of sterile cecal contents.

FIG. 6. Disk inhibition assay testing the sensitivity of B. fragilis strains to diamide in the absence of oxygen (bars with thick stripes) and in the presence of oxygen (bars with thin stripes) and to hydrogen peroxide (open bars). The cultures were grown overnight in BHIS, and 0.1 ml was plated on BHI agar without added cysteine. Ten microliters of a 2 M diamide solution or 0.88 M hydrogen peroxide was applied to a 6-mm filter paper placed in the center of each plate. Plates were incubated anaerobically overnight at 37°C. Duplicate plates with the diamide solution were exposed to air for 6 h at 37°C before they were placed in the anaerobic chamber and incubated as described above. Zones of growth inhibition (in millimeters) were measured. The error bars indicate the standard deviations of the means from three independent determinations. wt, wild type.

FIG. 7. Growth of B. fragilis strains in soft agar tubes in the presence oxygen. A 0.1-ml aliquot of bacteria grown overnight in BHIS was mixed with 5 ml BHIS without cysteine containing 0.4% agar at 45°C under anaerobic conditions. After solidification of the agar, cultures were placed outside the anaerobic chamber and incubated aerobically at 37°C for 24 h. The clearance zone was measured from the top edge of the agar down to the growth line in the column. The arrows indicate the interface between bacterial growth and the zone of growth inhibition. NG, no growth.
on the sensitivity of cells to oxidative stress in vitro. The lolA trxB operon appeared to be regulated at the transcriptional and posttranscriptional levels. At the transcriptional level the lolA trxB constitutive and stress-induced promoter regions (P1 and P2, respectively) do not have highly conserved consensus motifs with the previously described −10 and −35 sequence consensus in B. fragilis (4). This suggests that other regulatory promoter recognition mechanisms are involved in lolA trxB mRNA expression. Posttranscriptional regulation of polycistronic mRNA in B. fragilis has not been previously described and represents a new mechanism involved in oxidative and redox stress response regulation in this aerotolerant anaerobe. This regulation of the lolA trxB operon was unusual in that there was a greater abundance of trxB-specific mRNA than of the 1.8-kb bicistronic message. This was likely to be due to an increase in trxB mRNA stability following posttranscriptional modification of the lolA trxB mRNA since lolA trxB mRNA had a half-life of less than 1 min, while trxB single mRNA had a half-life for decay of around 25 min in the absence of oxygen (unpublished data).

The physiological roles of the Trxs in B. fragilis have not been established yet, but the observation that there are six Trxs suggests that there is a wide range of functions perhaps similar to those in photosynthetic cyanobacteria and plants, where Trxs have numerous roles (12, 24, 25, 35). Recent studies have shown that the Trx system is involved in the oxidative stress, metal homeostasis, and stationary-phase responses in other anaerobes (16, 22, 45) and in glycine metabolism in gram-positive anaerobic bacteria (1, 14). B. fragilis has a Trx peroxidase that is linked to survival during exposure to organic peroxides (16), and it is likely that the Trxs are involved in a variety of normal and stress response activities.

A classic role of Trx is as the hydrogen donor for class I ribonucleotide reductases (RRases) which are oxygen dependent. In a previous study we showed that B. fragilis possesses an oxygen-inducible class Ia RRase, NrdAB, which is important for survival during oxygen exposure because it allows cells to resume growth under anaerobic conditions after aerobic exposure (48). This is consistent with the fact that B. fragilis can resume growth and proliferate at the site of infection after the establishment of anaerobic conditions (42, 43, 44, 46). In many facultative bacteria, there are three major classes of RRases which vary in sensitivity and in the requirement for oxygen. Class Ia RRases are used for growth in the presence of oxygen, and class III RRases are used for growth in the absence of oxygen (21). The class II RRase is not affected by oxygen and can function independent of the presence of oxygen. Only class Ia (NrdAB) and class III (NrdDG) RRases are present in B. fragilis (48). Recently, it has been shown that in E. coli, which possesses all three classes of RRases, the class Ia enzyme, NrdAB, can be reduced by either Trx 1 or 2 or by glutaredoxin 1 (13). In this regard, we think that the TrxB/Trx system is likely to be the only reductive system linked to NrdAB induced in B. fragilis for anaerobic recovery following oxygen exposure or oxidative stress. This conclusion is based on our findings showing the lack of an alternative to the TrxB/Trx system.

The complexity of the thiol redox balance in B. fragilis is attributed not only to the presence of an extended number of Trx homologues but also to the presence of at least 13 additional thiol-disulfide oxidoreductase-like proteins with the CXXC motif and the Trx fold motif (data not shown). These proteins may play many roles in oxidation or reduction pathways in the cytoplasmic membrane or periplasmic space, similar to the Dsb proteins in E. coli (31). The observation that the gene encoding LolA, a prokaryotic periplasmic lipoprotein-specific molecular chaperone (27, 28), is cotranscribed with trxB in an operon regulated by oxidative and thiol redox stress suggests the idea that some of these proteins are localized in the periplasm and membrane of this anaerobe. Further studies should help us to clarify how B. fragilis TrxB, the Trxs, and thiol disulfide oxidoreductase-like proteins are employed to reduce different cytoplasm and periplasm substrates in an anaerobic organism.

The ability of B. fragilis to rapidly respond to an oxidative stress or a redox insult has been associated with its high aerotolerance compared to that of other anaerobic bacteria (29, 37). Even among different strains of B. fragilis the clinical isolates have been shown to be more aerotolerant than normal flora isolates (41, 51), and this characteristic has been assumed to be essential for their ability to survive in the more oxygenated host tissues, such as the peritoneal cavity, when they leave the anaerobic environment of the colon (37, 43). In this study, we showed that the B. fragilis trxB deletion mutant strain completely lost the ability to form intraabdominal abscesses in a mouse model. Recent reports have shown that Trxs and Trx reductase play an important role in the virulence and survival of pathogenic bacteria, yeasts, and protozoans (5, 26, 56) by contributing to the oxidative stress defense against toxic host cell-generated oxygen-derived radicals. In this regard, we show here that TrxB contributes to the survival of B. fragilis in the intraabdominal cavity, where the oxygen tension reaches 6% (33). One of the important roles of TrxB in the TrxB/Trx system during infection may be in the synthesis of deoxyribonucleotides in the presence of oxygen, or TrxB may contribute to cellular protection by reduction of the Trx peroxidase (16, 48). In addition, animal studies indicate that in the absence of functional TrxB, B. fragilis is unable to utilize host reduced thiol compounds as sources for thiol reducing power to overcome the growth deficiency in vivo. Further studies of this complex TrxB/Trx system should help clarify its role during normal growth in the colon and during adaptation in more aerobic extraintestinal sites.

ACKNOWLEDGMENTS

We thank Richard A. Franklin for assistance with the GSH/GSSG cycling enzyme assay utilizing glutathione reductase.

This work was supported in part by Public Health Service grant AI40588 to C.J.S. and by Public Health Service grant AI068659 to E.R.R.

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