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

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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 H_2O_2 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-*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Phenotype and/or genotype[a]	Reference or source
B. fragilis strains		
638R	Clinical isolate, Rif^r	34
IB298	638R Δ*oxyR*::*tetQ*	39
IB263	638R *oxyR*(Con), constitutively activating OxyR, hydrogen peroxide resistant	39
IB370	638R Δ*trxB*::*cfxA*	This study
IB372	638R Δ*oxyR*::*tetQ* Δ*trxB*::*cfxA*	This study
E. coli DH10B	Cloning host strain	Invitrogen
Plasmids		
pUC19	Cloning vector, (Amp^r)	Invitrogen
pFD340	*Bacteroides-E. coli* expression shuttle vector, (Sp^r) Erm^r	49
pFD516	Suicide vector, derived from deletion of pBI143 in pFD288, (Sp^r) Erm^r	50
pFD754	Internal 652-bp SalI/NdeI-blunted fragment of the *oxyR* gene was removed and replaced with a 2.1-kb SalI/SmaI *tetQ* gene in suicide vector pFd516	39
pFD846	Internal 60-bp BglII/ClaI fragment of the *trxB* gene was deleted and replaced with a 2.4-kb BamHI/NarI cefoxitin gene to construct a Δ*trxB*::*cfxA* deletion in pUC19	This study
pFD874	4.5-kb SphI/SstI fragment containing the Δ*trxB*::*cfxA* construct from pFD846 was cloned into the SphI/SstI site of the suicide vector pFD516	This study
pFD892	1,285-bp promoterless *trxB* fragment amplified from the 638R chromosome was cloned into expression vector pFD340	

[a] Erm^r, erythromycin resistance; Rif^r, rifamycin resistance; Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance. Parentheses indicate antibiotic resistance expression in *E. coli*.

ianum (36), *Porphyromonas gingivalis* (23), amino acid-utilizing anaerobic bacteria (14), *Clostridium acetobutylicum* and *Clostridium aminovalericum* (22), and *Chromatium vinosum* (20).

Although Grx/GSH-related genes have not been found yet in the genome sequences of strict anaerobes, a Grx homologue was a component of the AhpC peroxiredoxin reductase system in *C. pasteurianum* (36), and GSH has been found in crude extracts of *Desulfovibrio gigas* (11). In this regard, analysis of *B. fragilis* genome sequences (6) revealed the presence of a Trx reductase and six Trx homologues, but no Grx, γ-glutamyl-L-cysteine synthetase, or GSH synthetase genes were found. The large number of Trx orthologs in *B. fragilis* seems to be unusual compared to the smaller number of Trxs described in other anaerobes (14, 20, 22, 23, 36, 45).

B. fragilis clinical isolates are some of the most aerotolerant anaerobic bacteria (41, 51). Thus, we propose that the expanded Trx system is an important component of their arsenal against oxidative stress in vivo and in vitro. *B. fragilis* is the anaerobe isolated most often from clinical infections, and thus it is able to survive in oxygenated host tissues and withstand the oxidative burst of the host immune response during the initial steps of infection until appropriate anaerobic conditions that allow bacterial proliferation are present (42, 43, 44, 46). These findings prompted us to elucidate the role of the TrxB/Trx system in the physiology and aerotolerance of *B. fragilis* in vivo and in vitro. In this paper we present evidence showing that the TrxB/Trx system is the major thiol/disulfide redox system in *B. fragilis* and is essential for bacterial survival and abscess formation in an intraabdominal infection model.

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 $NaHCO_3$ (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. Semidefined 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: KH_2PO_4, 1.15 g/liter; NH_4SO_4, 0.4 g/liter; NaCl, 0.9 g/liter; L-methionine, 75 mg/liter; $MgCl_2 \cdot 6H_2O$, 20 mg/liter; $CaCl_2 \cdot 2H_2O$, 6.6 mg/liter; $MnCl_2 \cdot 4H_2O$, 1 mg/liter; $CoCl_2 \cdot 6H_2O$, 1 mg/liter; resazurin, 1 mg/liter; L-cysteine, 500 mg/liter; hemin, 5 mg/liter; and glucose, 5 g/liter. Twenty milliliters of 10% $NaHCO_3$ 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 L-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 Δ*trxB*::*cfxA* deletion in a new plasmid, pFD846/Δ*trxB*::*cfxA*. Then a 4.5-kb SphI/SstI fragment from pFD846/Δ*trxB*::*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-crossover allele exchange to create a *trxB* mutant designated IB370.

A Δ*trxB* Δ*oxyR* mutant was constructed by mobilizing suicide vector pFD754 (containing the Δ*oxyR*::*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/cefoxitin 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 IS*4351* 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 A_{550} of 0.3 and treated with 50 μM H_2O_2 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.

Primer extension analysis was performed with total RNA obtained from mid-log-phase cells of *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 [α-^{32}P]ATP 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 *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/oxidized 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 *B. fragilis* 638R and *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 then 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 −70°C until the assay was performed. Dried pellets were suspended in 800 μl of 2% 5-sulfosalicylic acid. GSH contents were determined using 100-μl samples, and immediately prior to the assay, samples were treated with 3.9 μl of a 1:4 dilution of triethanolamine to bring the pH to 7.0. The presence of GSSG was also determined in 100-μl samples by addition of 3.9 μl of 2-vinylpyridine prior to addition of 3.9 μl of a 1:4 dilution of triethanolamine. Briefly, the enzymatic assay was carried out using a 50-μl treated sample and a standard mixed with 100 μl of recycling reaction buffer containing 42.7 mM sodium phosphate (pH 7.5), 472 μM EDTA, 226 μM 5,5′-dithiobis(nitrobenzoic acid), 313 μM NADPH, and 20 U of glutathione reductase. Microplates were read at 405 nm every 45 s using a fluorescence/absorbance plate reader (FL600; Bio-Tek). Standard curves were used to determine the GSH/GSSG contents of the samples expressed in micromoles per gram (dry weight).

Mouse model of abscess formation. The experimental model for intraabdominal infection described previously was used, with minor modifications (7). *B. fragilis* 638R and the isogenic *trxB* mutant IB370 used for challenge were grown overnight in BHIS, concentrated, and then frozen until they were needed. The frozen cultures were titrated, and then just prior to use they were diluted to obtain the desired challenge dose. C57BL male mice that were 6 to 8 weeks old were challenged via intraperitoneal injection of 1×10^6, 1×10^7, or 1×10^8 cells in 0.2 ml (final volume) of BHI broth containing diluted sterile rat cecal contents. The mice were sacrificed 6 days later and examined for intraabdominal abscesses. The presence of one or more abscesses was considered a positive result. Animals inoculated with broth plus sterile cecal contents alone without bacterial challenge did not form abscesses.

RESULTS

Nucleotide sequence analysis of the TrxB/Trx system. The availability of the *B. fragilis* 638R genome sequence (6; http://www.sanger.ac.uk/Projects/B_fragilis/) allowed us to search for the presence of the Trx motif sequence. It was found that *B. fragilis* possesses an extensive Trx system consisting of a TrxB, six Trx orthologs, and more than 14 other proteins that were larger than Trxs but had the Trx-like protein fold and the CXXC motif (data not shown). The six Trx orthologs contain 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 *B. fragilis* Trx homologues were compared to other microbial Trxs using a progressive multiple alignment and parsimony analysis, it was found that except for TrxA, all *B. fragilis* Trx homologues were clustered together (data not shown). TrxA, however, grouped in a cluster together with *Flavobacterium*, *Corynebacterium*, and *Clostridium* Trx homologues.

The *B. fragilis* 638R *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 *B. fragilis* 638R TrxB was compared to other TrxBs, it showed the highest homology to *Bacteroides thetaiotaomicron* VPI-5482 TrxB (90% identity and 96% similarity) and *P. gingivalis* TrxB (84% identity and 80.5% similarity). Strong homology was also found to TrxBs of *E. coli* (52.2% identity and 62.3% similarity), *Haemophilus influenzae* (51.4 identity and 61.5% similarity), and *Mycobacterium tuberculosis* (49% identity and 59.3% similarity). In addition, the pyridine nucleotide disulfide oxidoreductase class II active site is conserved in *B. fragilis* TrxB (data not shown). The phylogenetic relationship between *B. fragilis* TrxB and 26 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 *B. fragilis* TrxB groups with other members of the unique *Cytophaga-Flexibacter-Bacteroides* phylum (see Fig. S1 in the supplemental material).

GSH determination. During analysis of the *B. fragilis* TrxB/Trx system, we noticed that there were no Grxs or any of the genes required for GSH biosynthesis (γ-glutamylcysteinyl synthetase and glutathione synthase) in the genome. This suggested that the TrxB/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 *B. fragilis* from mid-log-phase or stationary-phase cultures (limit of detection, 5 pmol in 50 μl of sample or standard). Cultures exposed to aerobic conditions also lacked detectable GSH/GSSG. In contrast, GSH/GSSG was present in the crude extracts of *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 *E. coli* under aerobic and anaerobic conditions (9).

Effect of free thiol on growth of a *trxB* mutant. A *trxB* mutant was constructed to determine if another mechanism was present that could maintain reduced cellular thiols and compensate for loss of the TrxB/Trx system. The availability of thiol compounds in the media greatly affected growth of the Δ*trxB* mutant in rich BHIS media and SDM. The Δ*trxB* 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 *trxB* strain to grow

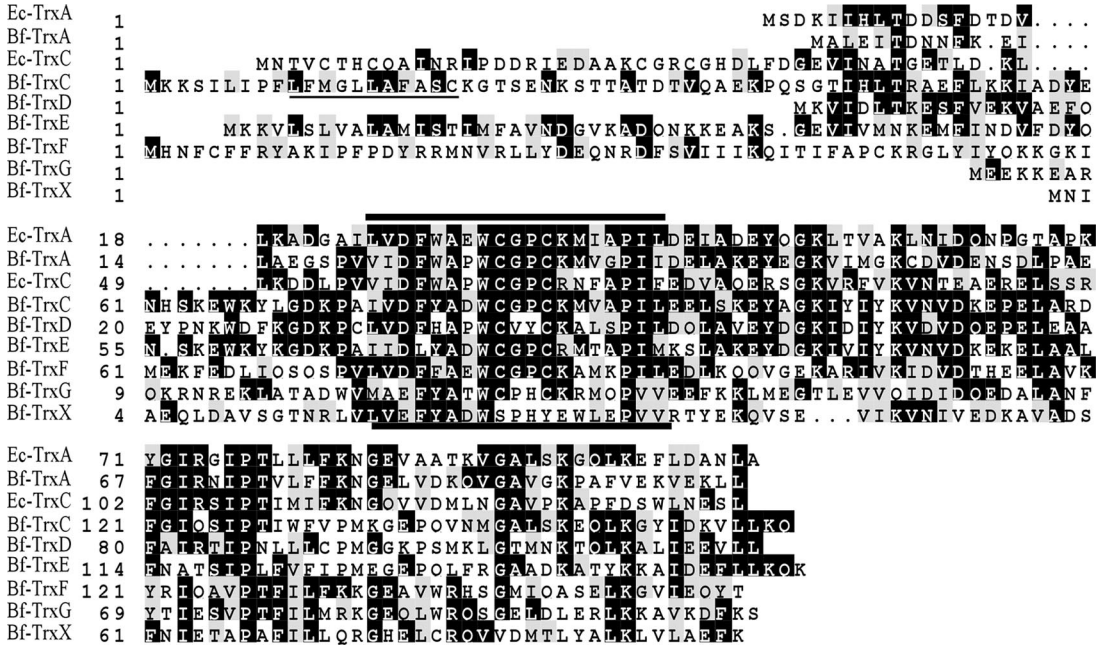

FIG. 1. Multiple alignment of the deduced amino acid sequences of *B. fragilis* (Bf) Trx orthologs (TrxA through TrxG plus TrxX) with *E. coli* (Ec) TrxA and TrxC amino acid sequences. The conserved Trx fold containing the CXXC redox active site is indicted by thick lines above and below the amino acid 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. 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 Δ*trxB* mutant would not have been so dependent on added thiol for growth.

Regulation of *trxB* expression by oxidative stress and diamide. 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 diamide was examined by Northern hybridization. The autoradiographs revealed that *trxB*

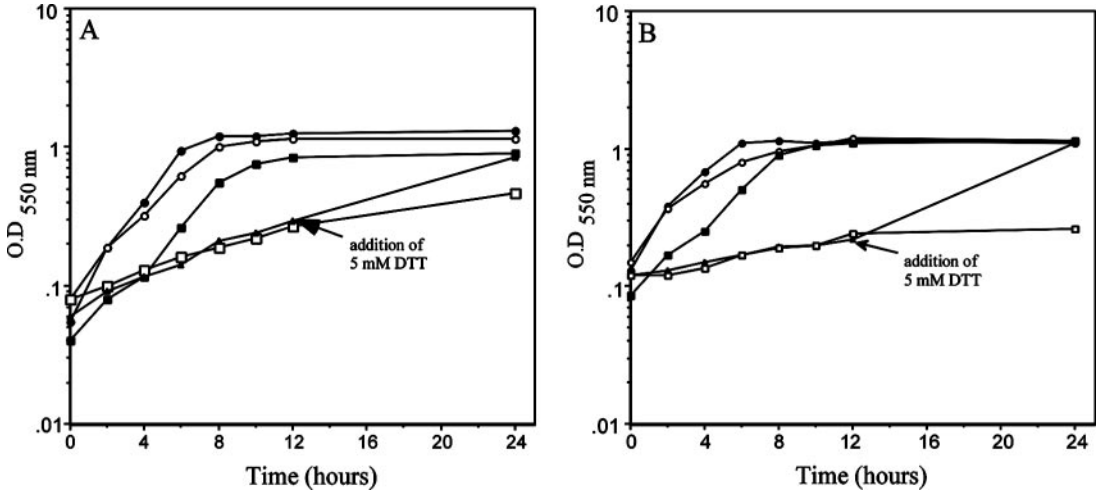

FIG. 2. Growth of *B. fragilis* parent strain 638R and the Δ*trxB* mutant in BHIS (A) and in SDM (B). ●, parent strain grown in medium containing cysteine; ○, parent strain grown in medium without cysteine; ■, *trxB* mutant grown in medium containing cysteine; □, *trxB* mutant grown in medium without 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.

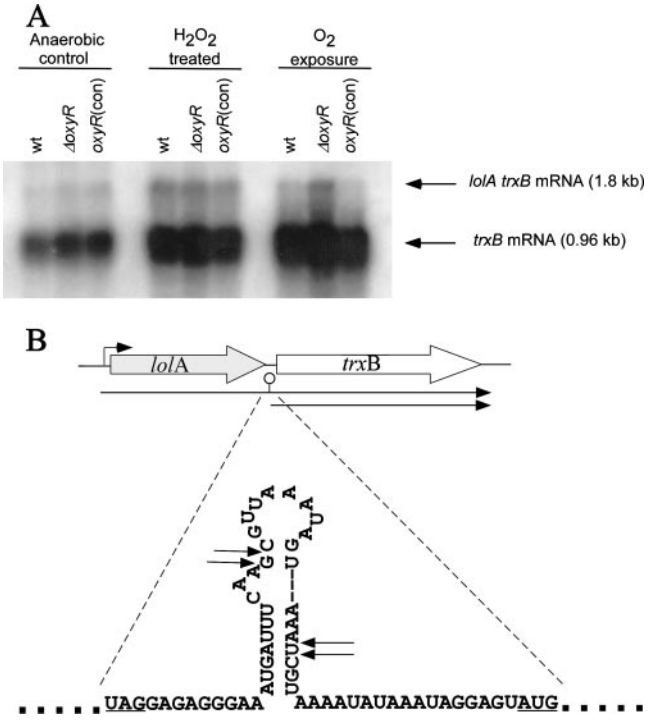

FIG. 3. (A) Northern hybridization analysis of total RNA of *B. fragilis* 638R (wt), IB298 (Δ*oxyR*), and IB263 constitutively activating OxyR [*oxyR*(Con)]. Cells were grown to mid-logarithmic phase in BHIS without addition of cysteine and then exposed to oxygen. Cultures were treated with H_2O_2 or exposed to oxygen as indicated in the text. The probe was a *trxB* internal gene fragment. The approximate sizes of the transcripts are indicated. DTT was used as a control reductant. (B) Diagram of *lolA* and *trxB* genetic organization and structure. The large arrows indicate the open reading frames and their direction of transcription. The arrows below the map indicate the lengths of the transcripts and their orientation. The bent arrow indicates the putative promoter predicted from data presented in Fig. 5. The *lolA trxB* intergenic nucleotide sequence region containing a predicted stem-loop structure is also shown. The *lolA* stop codon (UAG) and the *trxB* translational start codon (ATG) are underlined. The cleavage nucleotide regions of the bicistronic *lolA trxB* mRNA determined by the RNase protection assay are indicated by arrows.

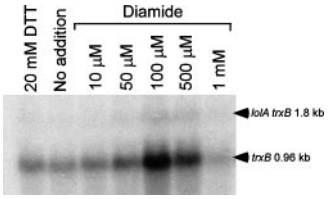

FIG. 4. Autoradiograph of Northern hybridization of total RNA from mid-log-phase *B. fragilis* 638R grown in BHIS without addition of cysteine. Cultures were treated with diamide at the concentrations indicated at the top. The probe was a *trxB* internal gene fragment. The approximate sizes of the transcripts are indicated. DTT was added to one culture as a control to show that addition of a reducing agent to the medium did not lead to induction of *trxB*.

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 anaerobic 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).

Primer 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 side. A *lolA* transcriptional fusion with the promoterless β-xylosidase 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 stain. 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

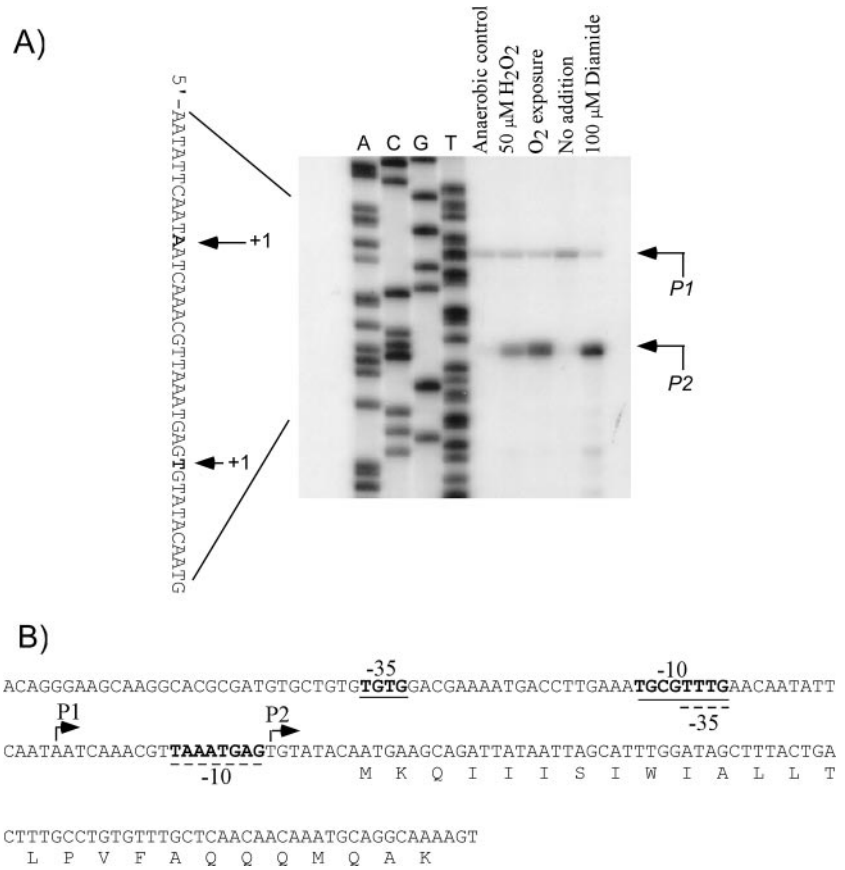

FIG. 5. Autoradiograph following primer extension analysis of the *lolA trxB* mRNA transcription initiation regions. Total RNA extracted from the wild-type strain under different stress conditions was used in the extension reactions. Cultures were treated by adding 50 μM H_2O_2, by exposure to atmospheric air for 1 h, or by adding 100 μM diamide. An untreated anaerobic control culture was also included. A DNA sequencing ladder generated with the same primer that was used for the primer extension reactions is shown on the left. The arrows indicate the initial +1 adenine for the P1 promoter and the +1 thymine for the P2 promoter. (B) Nucleotide sequence of the *lolA trxB* regulatory region. Based on a *B. fragilis* consensus (4), the predicted −10 and −35 promoter regions for the transcription initiation sites P1 and P2 are indicated by bold type and underlined with solid and dashed lines, respectively. The bent arrows indicate the initial +1 adenine nucleotide and the +1 thymine nucleotide for the P1 and P2 transcription start sites, respectively, at positions −26 and −8 relative to the translation start codon. The first 26 codons for *lolA* are shown.

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-

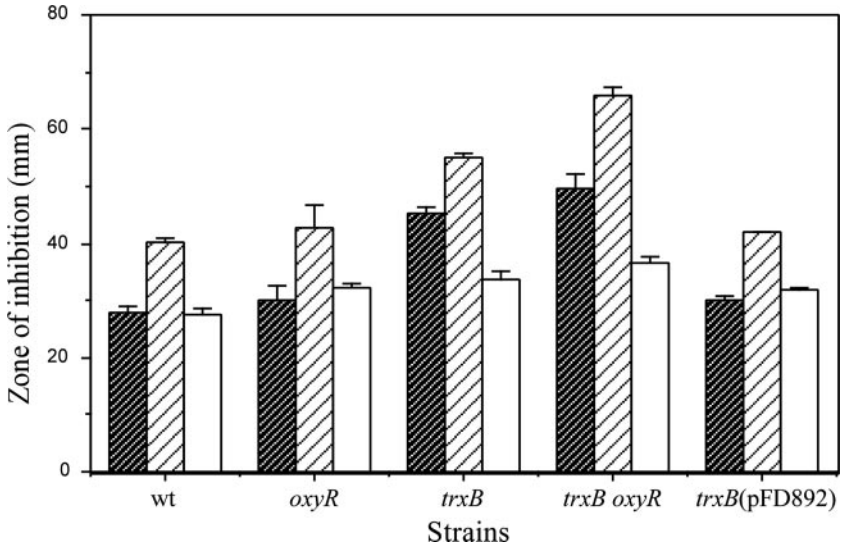

FIG. 6. Disk inhibition assay testing the sensitivity of *B. fragilis* strains to diamide in the absence of oxygen (bars with thick stripes) and in the presence of oxygen (bars with thin stripes) and to hydrogen peroxide (open bars). The cultures were grown overnight in BHIS, and 0.1 ml was plated on 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.

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

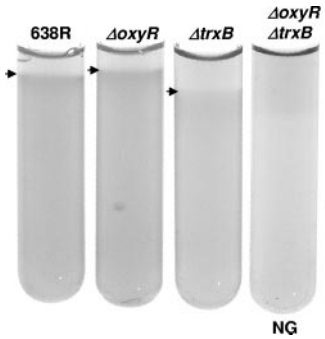

FIG. 7. Growth of *B. fragilis* strains in soft agar tubes in the presence oxygen. A 0.1-ml aliquot of bacteria grown overnight in BHIS was mixed with 5 ml BHIS without cysteine containing 0.4% agar at 45°C under anaerobic conditions. After solidification of the agar, cultures were placed outside the 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.

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

Strain	Intraperitoneal challenge (CFU)[a]	No. of animals with abscesses/ total no.	% of animals with abscesses
Wild type	1×10^8	8/8	100
	1×10^7	5/8	63
	1×10^6	2/8	25
trxB	1×10^8	0/8	0
	1×10^7	1/8	13
	1×10^6	0/8	0
Control[a]		0/8	0

[a] The control received only a 1:10 dilution of sterile cecal contents.

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 periplasmis 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 supports 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.

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